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THE FUNCTIONS OF THE OCELLI OF *CALLIPHORA* (DIPTERA) AND *LOCUSTA* (ORTHOPTERA)

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INTRODUCTION

Investigations into the visual capacities of the ocelli have been made by many previous workers, and various functions have been attributed to these organs. In the present work *Calliphora erythrocephala* Meigen and *Locusta migratoria* L. were employed to investigate the functions of the ocelli in form perception and as adjuncts to phototaxis. The optical powers of the ocellus were considered, and behaviour experiments were performed under critically controlled experimental conditions.

REARING OF MATERIAL

The culture of *Calliphora* was developed from a single batch of eggs in order as far as possible to obtain standardized insects. The locusts were offspring of gregarious parents and the methods of rearing were also standardized.

STRUCTURE OF THE OCELLUS

The biconvex lens of *Calliphora* is supported by a thin layer of corneagen cells containing minute pale brown pigment granules. The sensory layer lies immediately below the corneagen cells and is composed of elongate retinal cells. Rhabdoms were not identified with certainty, and it is therefore assumed that the retinal cells are sensitive to light along their entire length. The volume occupied by the sensory layer, 0·06 mm. deep, constitutes the retinal space (Fig. 1).

The lens of *Locusta* is also biconvex with a slight depression at the centre of the inner surface. The corneagen cells are extremely elongate and collectively they form a hemispherical body below the lens. Ovoid dark brown pigment granules are confined to the peripheral corneagen cells which curve inwards radially below the lens to form a pigment iris. The sensory cells are pear-shaped and aggregated in groups of four forming a distinct cup-shaped layer. Rhabdoms are again apparently absent, and the length of the sensory cells constituting the retinal space does not exceed 0·05 mm.

FORM PERCEPTION AND LIGHT-COLLECTING POWERS

The possibility of form perception by the ocellus depends on the ability of the lens to form an image, the location of the image plane relative to the retinal space and the ability of the sensory layer to receive and utilize the image so formed.

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If an ocellar lens of either the blowfly or the adult locust is removed and a drop of water placed on the inner surface of the lens is allowed to evaporate almost to dryness, well-defined inverted images may be seen through the microscope. Such images may be obtained of objects placed between 2 cm. and 5 m. from the lens of *Calliphora*, and less well-defined images obtained of objects placed as far as 1 m. from the lens of *Locusta*. If, however, the inner surface of the lens is perfectly dry it is impossible to obtain these images because the roughness of the inner surface of the lens scatters the light in all directions. It is reasonable to assume that the inner surface of the lens *in situ* is in contact with body fluid and that light rays passing through the lens are converged to a focus as in the isolated lens.

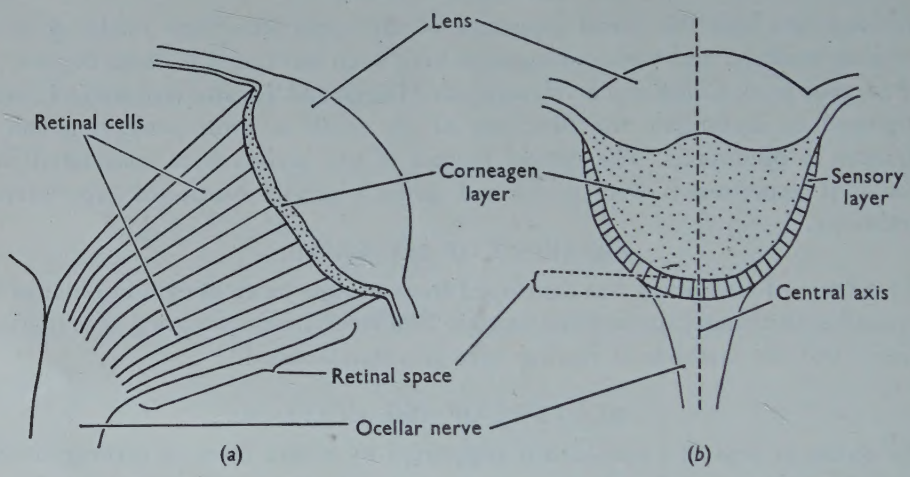

Fig. 1. Vertical section of the ocellus (diagrammatic). (*a*) *Calliphora*, (*b*) *Locusta*.

Parry (1947) showed that the principal focus of the ocellar lens of *Locusta* occurs 5 times as far behind the lens as the retina, and Homann (1924), working on a number of insects, showed that in no case were the focal and retinal planes coincident. Both these workers conclude that form perception by the ocellus is impossible.

The radius of curvature of the outer surface of the lateral and frontal ocellar lenses of *Calliphora* was measured by a modification of Homann's method (1924). From six determinations a mean value of 0·07 mm. ($\pm$ 0·004) was obtained. Using the same method for the adult locust, nineteen determinations provided the mean value of 0·47 mm. ($\pm$ 0·024). The radius of curvature for *Locusta* was also obtained graphically from medianly cut hand sections of lenses placed in a micro-projector. By this second method, twenty-five determinations provided the mean value of 0·44 mm. ($\pm$ 0·016). The mean obtained by these two methods in *Locusta* was therefore 0·46 mm. The refractive index of the ocellar lens was determined by Dethier's method (1942) and the values of 1·55 and 1·48 were obtained for *Calliphora* and *Locusta* respectively. The lenses were observed to be of uniform refractive index except at the periphery where slightly higher values were obtained.

Assuming that light refraction does not occur at the inner surface of the lens in contact with the corneagen layer the physical constants determined above provide focal length values of 0·12 mm. for *Calliphora* and 0·96 mm. for *Locusta*.

The distance of the outer limit of the retinal space from the outer surface of the lens of *Calliphora*, 0·04 mm., was ascertained by focusing the microscope first on to the outer surface of the lens, and secondly, focusing through the lens to the distal ends of the retinal cells. The measurements obtained from a vernier scale were then corrected for the difference in the refractive indices of air and the lens. Parry (1947), using this method for *Locusta*, apparently without the refractive index correction, said that 'the distance between the front of the lens and the retina was never found to reach the value of 0·09 mm.' In the present work, the mean thickness of the lateral and frontal ocellar lenses of the adult locust, ascertained from forty-six medianly cut sections, was found to be 0·18 mm. ($\pm$0·008). From sixteen determinations of the distance of the distal ends of the sensory cells from the inner surface of the lens, measured along the central axis, a mean of 0·27 mm. ($\pm$0·003) was obtained. The mean distance of the outer limit of the retinal space from the front of the lens of the locust was therefore 0·45 mm. The physical constants determined above provide means of 0·10 and 0·50 mm. for the distance of the inner limit of the retinal space from the outer surface of the ocellar lenses of *Calliphora* and *Locusta* respectively.

These investigations have shown that the plane of the principal focus of the ocelli of *Calliphora* and *Locusta* is located deeper in the ocellus than the limits of the retinal space. Light rays impinging upon the ocellar lens from objects close to the insect will converge to a focus even further outside the retinal space than parallel rays entering the lens from objects at infinity. Therefore, as the positions of the image space and retinal space are not coincident, the possibility of form perception of any degree of accuracy by the ocelli of these species must be excluded, and the ability of the sensory cells to receive and utilize the images formed need not be considered. It may be noted, however, that in both species, the centre of curvature of the outer surface of the lens occurs within the retinal space, but the implications of this are not clear.

The visual fields of the ocelli were investigated by observing the reflexion on the ocellar lens of a moving light source. When this reflexion was not obtained the source was known to be outside the visual field. This method provides the maximum possible field limited by the position of the ocelli on the head, and assumes that the sensory cells are stimulated by light incident anywhere on the lens. The presence of a cup-shaped sensory layer in *Locusta* and of retinal cells in *Calliphora* of which the distal ends are very close to, and have the same curvature as the hind surface of the lens, suggests that even oblique rays will be received. The wide visual fields of the ocelli (Figs. 2, 3) afforded by their positions on the head, and the structural arrangement of these organs support the suggestions of previous workers that the ocellus is essentially a light-gathering and not an image-forming organ.

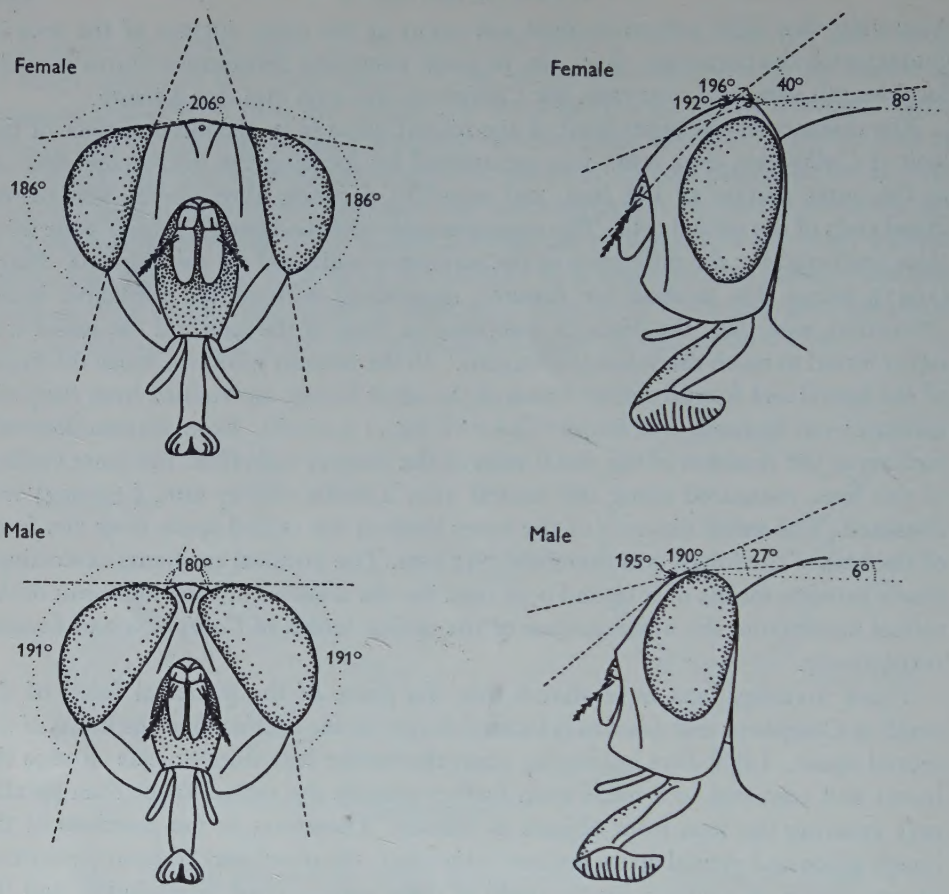

Fig. 2. Fields of view of the compound eyes and ocelli of *Calliphora*.

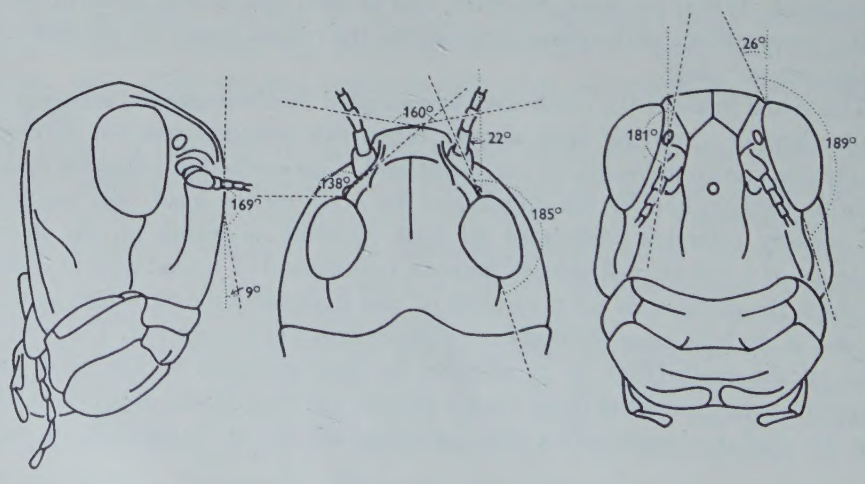

Fig. 3. Fields of view of the compound eyes and ocelli of an adult *Locusta*.

BEHAVIOUR EXPERIMENTS

Experimental conditions

Behaviour experiments with *Calliphora* were carried out at a constant temperature of 21° C. and relative humidity of 70%, and experiments with *Locusta* at a constant temperature of 27·5° C. and relative humidity of 75%. The room used was without windows and the four walls and ceiling were papered with a non-reflecting black paper. The bench was also covered with this paper, and as a further precaution the edges of the bench were boarded to a height of 6 in. with cardboard covered with black velvet. No electric light sources were permitted except those essential for the experiments, and in all cases these were screened by $2\frac{1}{2}$ in. thick water filters to obviate temperature effects. To remove reflexions, the glass filters were covered with black paper and all other apparatus was also blackened. The apertures of the light sources in experiments with *Calliphora* were 3·8 cm. with the centre at 5 cm. above the bench surface, and those in experiments with *Locusta* were 10 cm., with the centre 6·5 cm. above the bench surface. When recording tracks of walking insects, gloves and a coat of black material were worn to reduce reflexions from the hands and clothes.

Techniques

The part played by the ocelli in the phototactic reactions of *Calliphora* and *Locusta* was investigated by a series of experiments in which the effect of the occlusion of either the ocelli or compound eyes on the directional response was obtained. The eyes were occluded with an almost odourless and extremely quick-drying lacquer* which was applied to unetherized insects under a binocular microscope. In *Calliphora* there was no significant difference in the intensity of reaction between the sexes and as in the males the ocelli and compound eyes are very close together, only females were used in experiments where ocellar occlusion was required. A period of 30 min. was allowed after occlusion before starting an experiment, during which time the extra cleaning movements induced by handling and occlusion were effected. At the end of an experiment all insects were examined under a binocular dissecting microscope to check the complete occlusion of the sense organs. Results for insects with incomplete occlusion were discarded. In experiments made with walking flies the wing apices were removed and again a 30 min. period was allowed for the completion of cleaning activity. Locust hoppers were used to investigate walking reactions because adults showed a strong tendency to fly even with the wings severely clipped.

Phototactic reactions of Calliphora

It was first shown that *Calliphora* does not exhibit telotaxis. Two light sources of equal illumination and screened by water filters were placed 35 cm. apart, and paths made by intact flies released about 1 m. from the lights were seen to approximate to a line drawn symmetrically between the sources. Twenty intact insects

* 'Flinto Black', manufactured by Flinto Ltd., 157 Clarendon Road, London, W.C. 1.

were then released at the same distance from a single light source and in every case movement was made towards the source. On blackening one compound eye, repeated circus movements were made and from these observations it was concluded that *Calliphora* exhibits positive photo-tropotaxis.

To investigate the phototactic reaction of *Calliphora*, twelve normal flies were introduced into a 15 cm. Perspex cube on which a line was drawn dividing the cube into two equal halves. Random distribution of the flies was allowed and with incident light from above, the number of flies in the upper half was noted at intervals of 30 sec. The ocelli were occluded and the experiment was repeated. The procedure was again repeated subjecting specimens with ocelli functional and occluded to incident light from below and also from one side. The intensity of reaction was calculated as a percentage of the flies present in that half of the cube nearer to the light source. The results shown in Table 1 indicate that when the ocelli are occluded there is a small decrease in the intensity of reaction for all directions of incident light. The difference between the intensities of reaction to incident light from above and below is attributed to the association of the positive phototactic response with a negative geotactic response. To investigate more critically the effect of occluding the ocelli on the phototactic response, the following experiment was conducted in which the length of path made by walking flies was recorded.

An insect which exhibited a completely efficient phototactic response would move in a straight line towards a light source. By measuring with an opisometer the actual distance travelled along a curved path (Fig. 4), and comparing this with the length of the straight line, a measure of the 'efficiency' of response is obtained. Paths of males and females with functional ocelli were measured, and the results shown in Table 2 indicate that there was no significant difference between the efficiency of movement of the sexes. Females only were used when path measurements were made of flies with occluded ocelli. Using a bright light source occlusion of the ocelli produced a significant decrease in the efficiency of response. Paths made by females with functional and occluded ocelli are represented to scale in Fig. 4. The angular paths made with occluded ocelli resulted from the two modes of progression shown in Fig. 5. When a less luminous source was used no significant difference between the efficiency of response of normal and anocellate insects was obtained. With this source the paths and modes of progression made with functional and occluded ocelli resembled those made by flies with occluded ocelli when subjected to bright illumination.

The next experiment was designed to investigate the effect of ocellar occlusion on the phototactic response to a change in direction of incident light. Two light sources screened by water filters were placed at the positions *B* and *D* as shown in Fig. 6. Flies released at *A* were allowed to walk towards the illuminated source *B*, but on reaching *C* the source *B* was extinguished and source *D* simultaneously illuminated. The insects continued along curved paths reorientated to the second source. The points *E* on the curves farthest from the line *CD* were recorded and their distance from the line *CD* was measured. These operations were conducted

on females, first with functional ocelli, and secondly with ocelli occluded. The light source *B* orientates the fly at *C* so that on the illumination of source *D* only one compound eye is subject to stimulation. Measurements were made only on flies

Table 1. *The effect of occluding the ocelli on the intensity of reaction of flies aggregating towards a light source*

Experimental conditions:	Temperature 21° C. Relative humidity 70%. Illumination provided by a 40 W. bulb					
Direction of incident light:	Above		Below		Side	
Condition of ocelli:	Functional	Occluded	Functional	Occluded	Functional	Occluded
Number of 30 sec. periods	10	14	17	17	14	28
Intensity of reaction (%)	95	85·8	75	67·5	85·8	79·2
Decrease in reaction (%)	9·2		7·5		6·6	

Table 2. *The effect of occluding the ocelli on the efficiency of the phototactic response of walking* Calliphora

Experimental conditions:	Temperature 24° C. Relative humidity 70%					
	Bright illumination Illumination at *A* $\bar{1}$·16, *B* 0·30 (log. ft. lamberts)			Dim illumination *A* $\bar{2}$·66, *B* 0·17 (log. ft. lamberts)		
Condition of ocelli:	Functional		Occluded	Functional		Occluded
Sex:	♂	♀	♀	♂	♀	♀
Number of specimens	27	39	23	36	41	31
Mean length of path (cm.)	62·4	63·3	65·0	65·5	64·3	65·5
Efficiency of phototaxis (%)	97·8	96·4	93·8	93·1	94·8	93·1
t	1·24 non-sig.			1·92 non-sig.		
	2·80 ($P<0·01$)			1·74 non-sig.		

Positions *A* and *B* refer to the starting and finishing positions, respectively, of the paths shown in Fig. 4.

which travelled without stopping from the starting position to source *D*. Occasionally, on the simultaneous changing of the sources a fly at *C* momentarily became stationary, turned axially through an angle of 90 degrees and then proceeded towards source *D*. Records of flies effecting such movements were not included.

Table 3 shows that when *Calliphora* is subjected to a change in the direction of incident light there is a significant decrease in the efficiency of reorientation by flies with occluded ocelli. The average paths effected by insects with ocelli functional and occluded have been represented in Fig. 6.

Finally, the role played by the ocelli in the phototactic response was investigated by occluding both compound eyes and subjecting flies with only the ocelli functional to a single light source. The erratic paths made by walking flies released facing in varied directions and at varied distances from the light source are shown in Fig. 7. From this experiment it may be concluded that phototactic orientation by means of the ocelli alone is impossible.

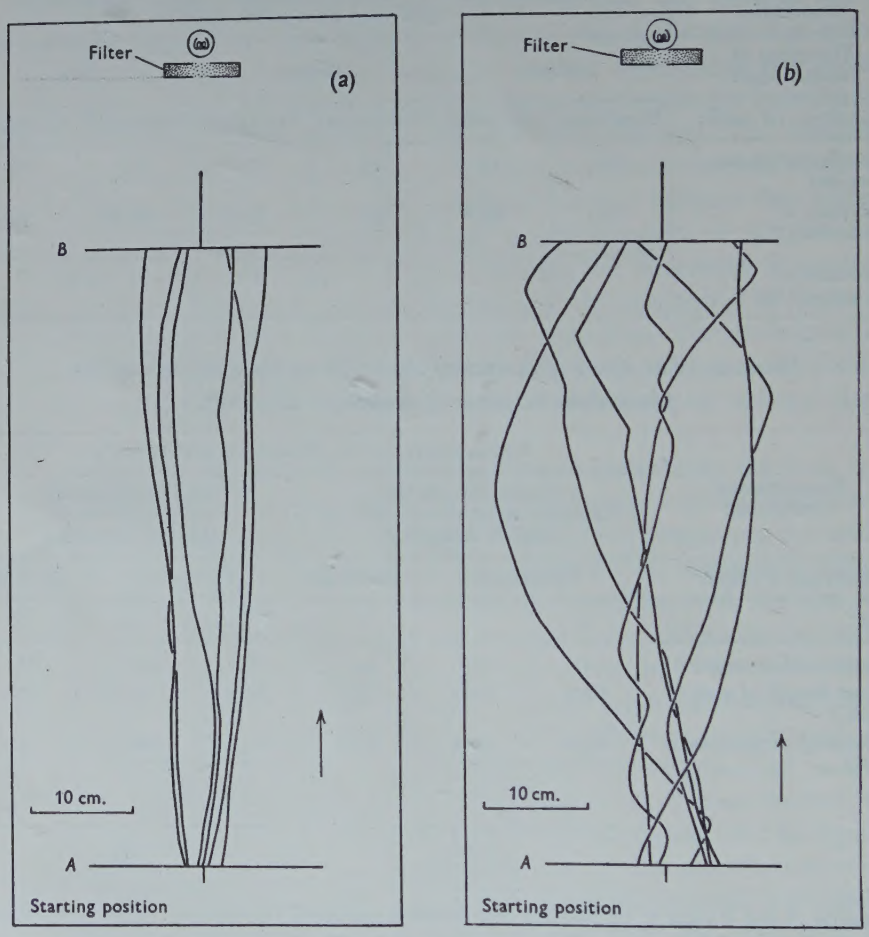

Fig. 4. (*a*) Paths made by walking *Calliphora* with the compound eyes and ocelli functional. (*b*) Paths made by walking *Calliphora* with the compound eyes functional and the ocelli occluded.

These experiments have shown that under laboratory conditions the ocelli of *Calliphora* are incapable of mediating a directional response and that with the ocelli occluded there is a decrease in the efficiency of phototactic movement and reorientation to a change in direction of incident light. These results are in agreement with observations of previous workers. That insects with the compound eyes blackened behave as if blind has been shown by Brandt (1937), with *Drosophila* by

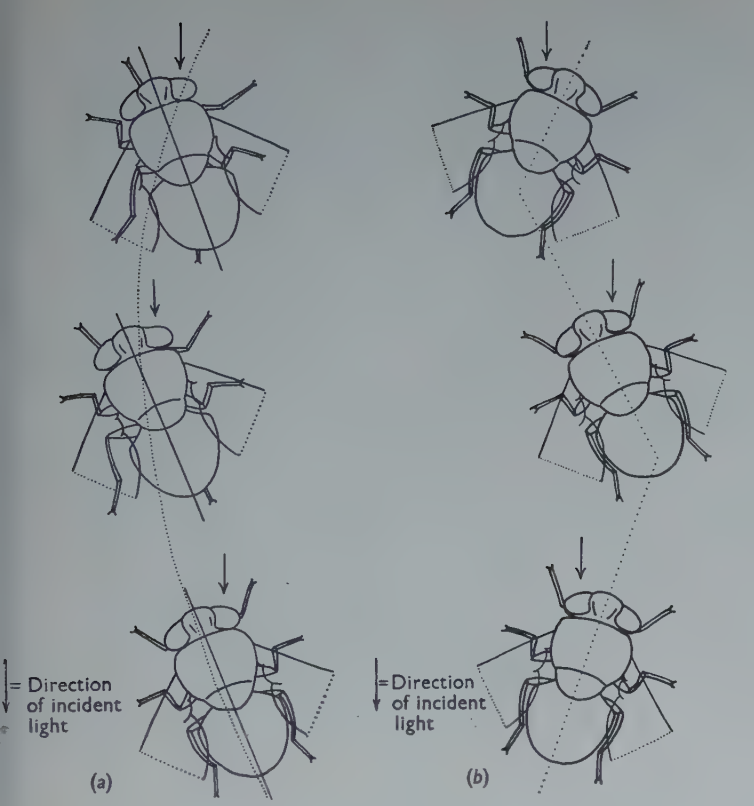

Fig. 5

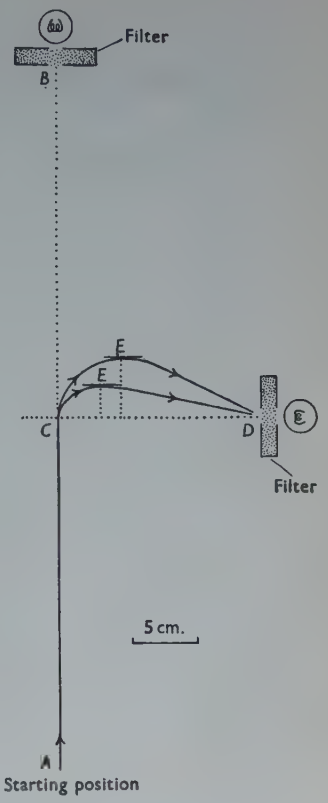

Fig. 6

Fig. 5. (*a*) Mode of progression of walking *Calliphora* with the ocelli occluded. (*b*) Mode of progression of walking *Calliphora* with the ocelli occluded.

Fig. 6. The phototactic response of *Calliphora* to a change in direction of incident light.

Table 3. *The effect of occluding the ocelli of* Calliphora *on the phototactic response to a change in direction of incident light*

Experimental conditions:	Temperature 21° C. Relative humidity 70 %. Illumination at C produced by source $B = 0{\cdot}27$. Illumination at C produced by source $D = 0{\cdot}70$. (log. ft. lamberts)	
Condition of ocelli:	Functional	Occluded
Number of specimens	19	14
Mean distance of tangent at E from line CD (mm.)	25·0	42·6
t	4·14 ($P < 0{\cdot}001$)	

Position C and light sources B and D refer to Fig. 6.

Bozler (1926), with bees by Müller (1931) and with ants by Homann (1924). Wellington (1953), however, showed that with low light intensity in the laboratory, *Sarcophaga* with the ocelli alone functional exhibited undirectional movement, but outdoors under conditions of high light intensity such insects produced movements as directed as any made by flies with the compound eyes functioning. Further, he

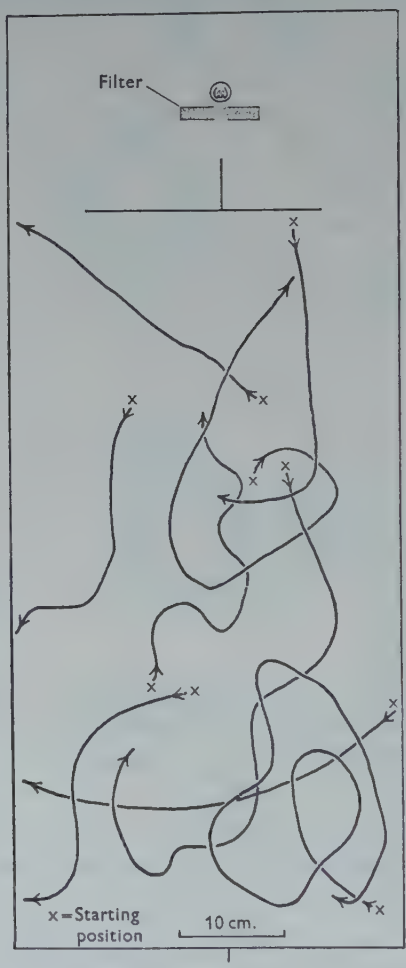

Fig. 7. Paths made by walking *Calliphora* with the compound eyes occluded and the ocelli functional.

showed that the ocelli of adult Sarcophagid flies are sensitive to changes in polarized light from the sky. Von Buddenbrock (1937) and Kalmus (1945) suggested that the ocelli aid in orientation during flight, and that they assist in the phototactic orientation of bees and ants has been suggested by Müller (1931). At low light intensities Bozler (1926) established that ocellar occlusion resulted in a slight reduction of the 'normal phototactic orientation potential'.

In bright illumination, the decrease in the efficiency of the directional response

of *Calliphora* with occluded ocelli suggests that there is a correlation of function between the ocelli and compound eyes. In dim illumination there was no marked decrease in the efficiency of response which suggests that in *Calliphora* there is a threshold of light intensity below which the ocelli are not sensitive. In bright illumination, the progression of normal flies was such that the longitudinal axis of the insect was alined with the steepest light gradient and corresponding parts of both compound eyes were equally stimulated. With the ocelli occluded, two types of progression were exhibited during which the axis of the insect was not alined with the steepest light gradient, but at an angle to it, and there was a failure for the corresponding parts of the two eyes to be stimulated. The curved path shown in Fig. 5(*a*) appears to be the result of a turning reflex evoked by stimulation of the antero-lateral region of one compound eye. In Fig. 5(*b*) it appears that as the result of too great a turn the antero-lateral region of the other eye becomes stimulated and this eye continues to mediate turning reflexes. Continued progression of this type results in the formation of a zigzag path, and movement towards the light source may result from either one or both these modes of progression. When a fly is subjected to a change in direction of incident light and the lateral region of one compound eye is stimulated, a turning movement is mediated by the lateral ommatidia followed by a direct path to the source resulting from equal stimulation of the anterior regions of both compound eyes. When the ocelli are occluded, however, orientation appears to be the result only of a reflex turning movement mediated by the lateral ommatidia, producing a less efficient path to the source.

Phototactic reactions of Locusta

First-instar nymphs were subjected to a single light source and paths made by walking hoppers are represented in Fig. 8. There appears to be no uniformity in the direction of walking, and from these observations it was impossible to define the normal phototactic response of *Locusta*. As similar results were obtained with all nymphal stages the following experiment was designed.

A grid of ninety-six 2 in. squares was drawn on a piece of black paper (Fig. 9), and a light source and filter placed at one end provided the light intensities as shown. One hundred and fifty hoppers (seventy-five first-instar and seventy-five second-instar nymphs) were released one at a time at the opposite end of the grid directly facing the source, and paths were recorded by noting the squares through which the hoppers walked. The table of frequency obtained has been represented as a density diagram which shows that there is a tendency for the hoppers to walk either towards the source or at right angles to it. The latter type of orientation is a 'light compass reaction' as was observed by Kennedy (1945).

The next experiment was conducted to explain the existence of two types of light reaction in the same insect. Three fifth-stage nymphs were subjected to bright illumination at a temperature of 36–40° C. for a period of half an hour. Repeated trials were made with each specimen using the light source as described in the above experiment, and the paths shown in Fig. 10 indicate that in all cases hoppers progressed towards the source. Detailed examination, however, shows that in

many cases the paths are at a constant acute angle with the direction of incident light, and in repeated trials with the same specimen this angle is approximately preserved. The same three specimens were then placed in complete darkness for half an hour at the same temperature, and repeated trials were again made. The

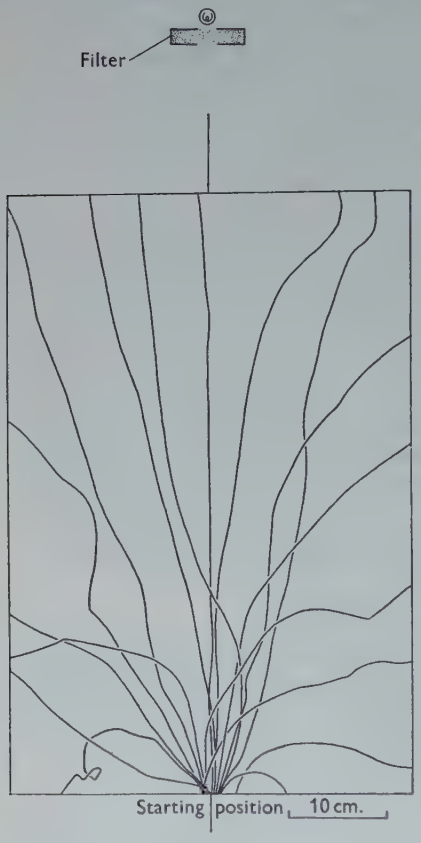

Fig. 8

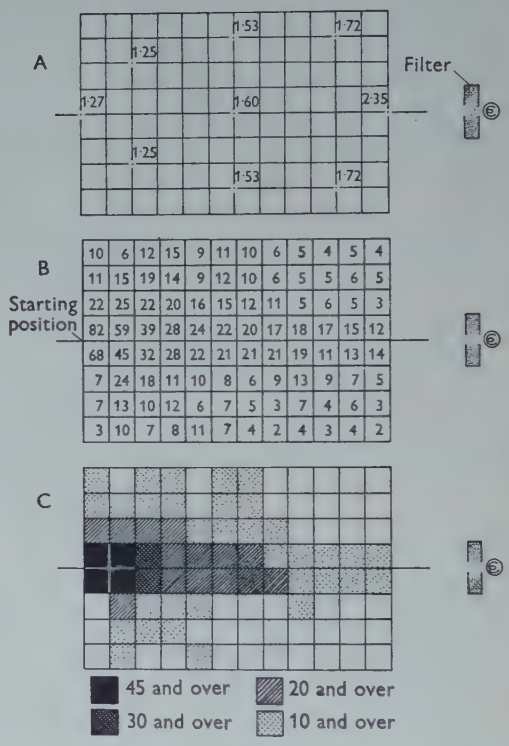

Fig. 9

Fig. 8. The phototactic response of first-instar nymphs of *Locusta*.

Fig. 9. *Locusta* first- and second-instar nymphs. See text. (A) Light intensities recorded in log. ft. lamberts. (B) Frequency with which squares were entered by hoppers. (C) Figures of (B) grouped.

paths shown in Fig. 11 indicate that under these conditions hoppers exhibit a light compass reaction. Locusts released facing the source progress slightly towards it, turn, and continue at right angles to it. Those released facing at right angles to the light continue to walk approximately in this direction.

It was concluded from the results of this experiment that the direction of path made by a walking hopper depends on its state of photic adaptation. Dark-adapted specimens progressed approximately transversely to a light source with fixation of the stimulus by a set of ommatidia in the lateral region of one eye, and light-adapted

hoppers progressed towards the illuminating source with fixation of the stimulus in the anterior regions of both eyes.

The next experiment was designed to ascertain the effect of occluding the ocelli on the directional response. Two sources (with water filters) emitting light of the same intensity were placed at opposite sides of a circle. The circumference was divided into seventy-two equal portions each corresponding to an angle of five degrees (see Fig. 12). Four fifth-stage nymphs were dark-adapted for a period of 1 hr. and each was subjected to the illumination of the two light sources in the following manner. With one source illuminated and the other extinguished, a hopper was placed at the centre of the circle, first facing the source (i.e. at 0 degrees),

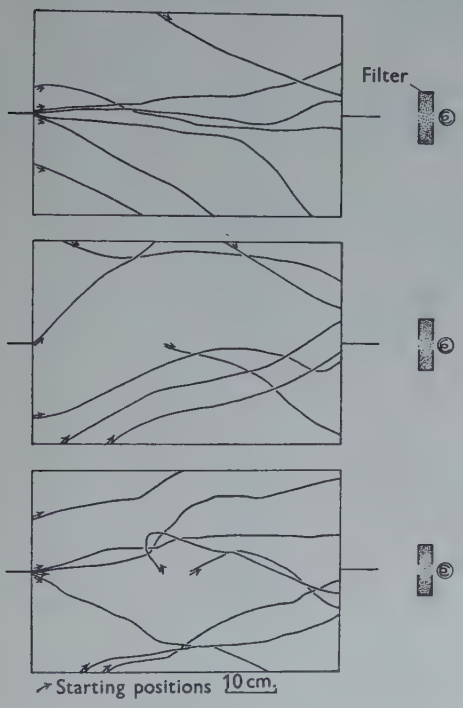

Fig. 10

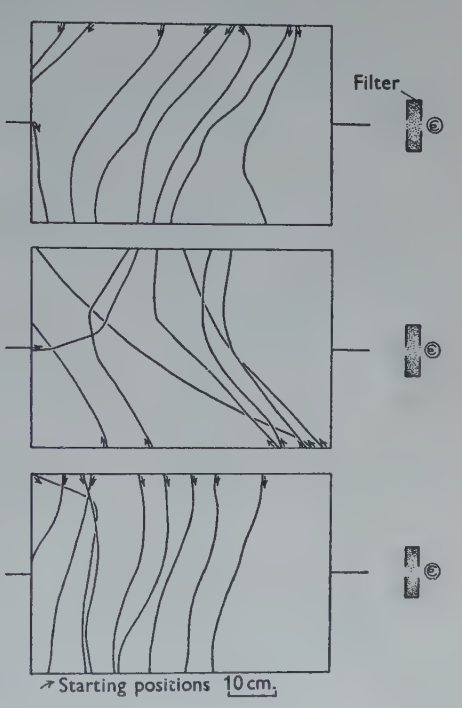

Fig. 11

Fig. 10. Paths made by light-adapted fifth-instar nymphs of *Locusta*.

Fig. 11. Paths made by dark-adapted fifth-instar nymphs of *Locusta*.

then at right angles to the source (i.e. at 90 degrees), thirdly facing away from the source (i.e. 180 degrees) and lastly again at right angles (i.e. 270 degrees). The angle of the point at which the insect crossed the circumference of the circle was recorded, and this procedure was repeated 5 times with one source illuminated and then 5 times with the second source illuminated. Four presentation angles were used because of the difference in response in the two conditions of adaptation. Two light sources were used to eliminate any undesirable lighting effects inherent in the apparatus. The same four hoppers were light-adapted for 1 hr. between four

40 W. bulbs situated so as to ensure equal adaptation of both eyes, and they were then subjected to the procedure described above for the dark-adapted state. Following this, the ocelli were occluded, the insects were again dark-adapted for

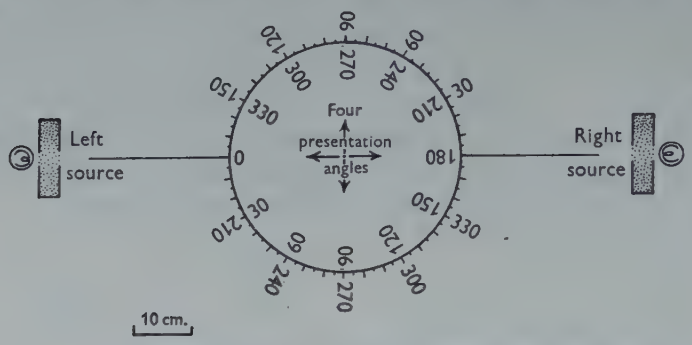

Fig. 12. Design of experiments to investigate the phototactic reactions of *Locusta* hoppers.

Table 4. *The effect of occluding the ocelli on the directional response of fifth-instar* Locusta *nymphs after dark- and light-adaptation*

Condition of ocelli:		Normal				Occluded			
Presentation angle:		0°	90°	180°	270°	0°	90°	180°	270°
Mean angle of 'deviation from parallelism' after dark-adaptation									
Specimen	Source								
1	Right	80	68	39	58	66	59	42	50
	Left	33	41	58	78	53	28	48	58
2	Right	75	79	52	46	77	85	63	41
	Left	67	49	28	43	82	57	45	46
3	Right	42	55	25	67	62	43	37	56
	Left	57	50	35	69	65	67	51	50
4	Right	61	78	53	60	81	75	23	29
	Left	30	50	56	36	59	48	82	72
Mean		53·7				56·3			
Mean angle of 'deviation from parallelism' after light-adaptation									
1	Right	23	24	22	18	19	26	47	28
	Left	18	11	35	33	25	17	33	25
2	Right	6	12	31	23	33	55	80	53
	Left	27	12	21	42	41	46	39	40
3	Right	29	60	67	50	64	63	50	77
	Left	7	22	29	13	43	35	70	57
4	Right	21	18	48	34	50	72	62	53
	Left	17	41	35	18	49	34	50	42
Mean		27·1				46·2			

an hour and further observations were recorded. Finally, with the ocelli occluded, the specimens were again light-adapted and a final series of observations was made.

The results of this experiment are summarized in Tables 4 and 5, and analyses of variance were made to ascertain whether photic adaptation or ocellar occlusion

influences (*a*) the 'positiveness of phototaxis' and (*b*) 'deviation from parallelism'. The values of 'positiveness of phototaxis' were obtained by ascribing a value of 1 to each positive observation and a value of $\frac{1}{2}$ to each observation of 90 or 270 degrees. The mean angle of 'deviation from parallelism' was calculated after converting the observed angles to their equivalents lying between the limits of 0 and 90 degrees. Paths towards the source at angles of 30 or 330 degrees, or away from the source at 150 or 210 degrees are thus both equivalent to 30 degrees when considering 'deviation from parallelism'.

Table 5. *The effect of occluding the ocelli on the directional response of fifth-instar* Locusta *nymphs after dark- and light-adaptation*

Condition of ocelli:		Normal				Occluded			
Presentation angle:		0°	90°	180°	270°	0°	90°	180°	270°
		'Positiveness of phototaxis' after dark-adaptation							
Specimen	Source								
1	Right	2·5	0·5	0·0	1·0	0·0	0·0	0·0	1·5
	Left	5·0	5·0	2·0	4·0	3·5	3·0	1·0	4·0
2	Right	1·0	0·0	0·0	0·0	5·0	4·5	0·0	1·0
	Left	0·5	0·0	0·0	0·0	2·0	1·0	1·0	0·0
3	Right	1·0	0·5	0·0	0·0	1·0	0·0	0·0	0·0
	Left	3·0	2·0	0·0	0·5	2·0	1·5	0·0	0·0
4	Right	4·0	2·5	1·0	2·0	2·5	1·0	0·0	0·5
	Left	5·0	4·0	1·0	1·0	5·0	5·0	5·0	4·0
Mean		1·53				1·72			
		'Positiveness of phototaxis' after light-adaptation							
1	Right	5·0	5·0	4·0	5·0	5·0	5·0	3·0	5·0
	Left	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0
2	Right	5·0	5·0	5·0	5·0	5·0	5·0	1·0	4·0
	Left	5·0	5·0	5·0	5·0	5·0	4·0	2·0	1·0
3	Right	5·0	3·0	1·0	2·0	1·0	3·5	1·0	0·0
	Left	5·0	5·0	5·0	5·0	4·0	4·5	3·0	4·0
4	Right	5·0	5·0	5·0	5·0	5·0	1·0	1·0	2·0
	Left	5·0	5·0	5·0	5·0	4·0	5·0	4·0	4·0
Mean		4·69				3·50			

The analyses of variance showed that there was a significant effect of adaptation ($P<0{\cdot}001$) on the 'positiveness of phototaxis' and angular direction of path. Furthermore, the significance of the interaction between the state of adaptation and condition of ocelli ($P<0{\cdot}001$ for 'positiveness of phototaxis', $P<0{\cdot}01$ for 'deviation from parallelism') showed that hoppers with their ocelli normal or occluded behaved differently in the two states of adaptation. Separate analyses of variance for each state of adaptation showed that occlusion of the ocelli produced a significant effect only in the state of light-adaptation, decreasing the positiveness of the reaction and increasing the angular 'deviation from parallelism'.

In a repetition of this experiment using five third-instar nymphs each hopper was subjected to the stimulation of one light source 10 times at each of the presenta-

tion angles. As ocellar occlusion was found to have an effect only after light-adaptation no observations were made for the dark-adapted state. The sequence of dark- and light-adaptation and of ocellar occlusion as described in the previous experiment was however retained. Statistical analysis of the results shown in Table 6 indicated that occlusion of the ocelli increased the angular 'deviation from parallelism' ($P < 0{\cdot}01$), but appeared to have no significant effect on the 'positiveness of phototaxis'. These results thus only partly confirmed the results of the previous experiment using fifth-stage nymphs.

Table 6. *The effect of occluding the ocelli on the directional response of third-instar* Locusta *nymphs after light-adaptation*

Condition of ocelli:	Normal				Occluded			
Presentation angle:	0°	90°	180°	270°	0°	90°	180°	270°
	Mean angle of 'deviation from parallelism'							
Specimen 1	10	20	36	10	16	42	31	20
2	24	47	33	14	11	38	52	23
3	20	24	22	17	24	38	35	27
4	9	14	31	29	29	29	45	49
5	9	14	8	24	7	9	3	48
Mean	20·8				28·8			
	'Positiveness of phototaxis'							
1	10·0	10·0	5·5	10·0	10·0	9·0	2·0	9·0
2	10·0	5·5	4·0	10·0	10·0	9·0	3·5	8·0
3	10·0	10·0	8·0	10·0	10·0	10·0	8·0	8·0
4	10·0	10·0	8·0	10·0	8·0	10·0	8·0	9·0
5	10·0	10·0	10·0	10·0	10·0	10·0	8·0	10·0
Mean	9·05				8·48			

Final experiments were made to investigate the part played by the ocelli in phototaxis when the compound eyes are occluded. Hoppers with the ocelli alone functional were placed at varied distances from, and at different angles to, a source of illumination. Their paths shown in Fig. 13 were found to have a strong resemblance to those made by completely blind hoppers. To confirm this observation the following experiment was designed.

The compound eyes of four fifth-stage nymphs were occluded and the specimens were dark-adapted for 1 hr. With the apparatus arranged as described previously, each hopper was placed at the centre of the circle seven times at each of the four presentation angles and subjected to the illumination of a single light source. The angle of response was recorded, the hoppers were then light-adapted for 1 hr. and further trials were recorded. Lastly, the ocelli were occluded and the hoppers, now completely blind, were again subjected to stimulation. Analysis of variance of the results shown in Table 7 indicated that there was no significant difference between the reactions of blind hoppers and of those with the ocelli alone functional in the light- or dark-adapted states. When the only form of stimulation is a light source,

blind specimens will exhibit no directional response. Movement will be random and the mean angle of 'deviation from parallelism' will be 45 degrees. A final statistical test was made to ascertain whether the experimental values obtained with the ocelli alone functional differed significantly from this expected mean. A non-significant probability was obtained, indicating that the hoppers behaved as if blind.

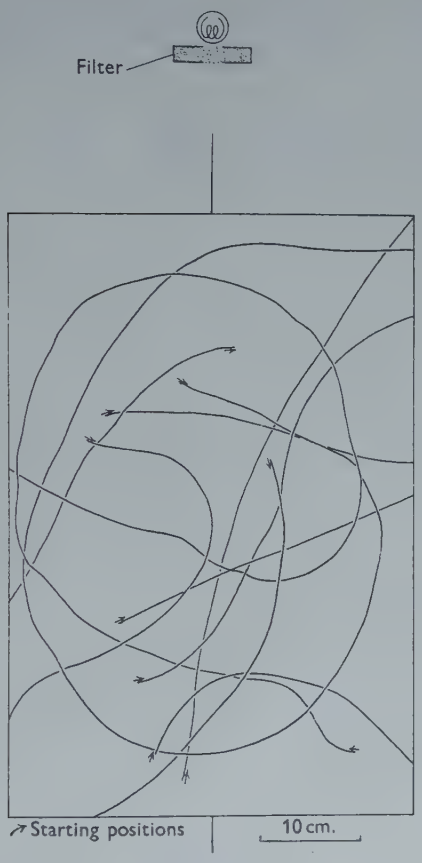

Fig. 13. Paths made by fifth-instar nymphs of *Locusta* with the compound eyes occluded and the ocelli functional.

These experiments have shown that the phototactic response of walking hoppers depends on their state of photic adaptation. Dark-adapted hoppers exhibit a light compass reaction and walk approximately at right angles to a light source, with fixation of the stimulus by a set of ommatidia in the lateral region of one compound eye. Light-adapted hoppers progress towards the source with fixation of the stimulus in the anterior regions of both compound eyes. It is difficult to explain the mechanism responsible for these two types of orientation. It has been shown (Roeder, 1953) that light-adaptation may be described as a loss in sensitivity of the eye and that an increase in light intensity is required to cause recurrence of a response. Presumably light-adaptation in the locust results in a decrease in the

sensitivity of the compound eyes, the lateral ommatidia are then unable to fix the source and a response may be mediated only by the more sensitive ommatidia of the anterior regions of both compound eyes. Occlusion of the ocelli was shown to decrease the efficiency of orientation only when the sensitivity of the eye is decreased by light-adaptation and when a response is evoked by the stimulation of the ommatidia of the anterior regions of the compound eyes. When the compound eyes are occluded, the ocelli alone are not capable of mediating a directional response under the light conditions of the laboratory, and the insects behave as if blind.

Table 7. *The effect of occluding the ocelli on the directional response of fifth-instar* Locusta *nymphs when the compound eyes are also blackened*

Condition of compound eyes:	Occluded				Occluded				Occluded			
Condition of ocelli:	Occluded				Normal				Normal			
Type of adaptation:	—				Dark				Light			
Presentation angle:	0°	90°	180°	270°	0°	90°	180°	270°	0°	90°	180°	270°
	Mean angle of 'deviation from parallelism'											
Specimen 1	33	56	45	31	16	31	52	58	20	51	45	71
2	36	46	54	51	38	59	49	39	44	37	50	60
3	26	66	41	59	49	60	53	56	33	64	35	44
4	51	40	59	51	39	44	36	70	66	48	60	38
Mean	46·6				46·8				47·9			
	'Positiveness of phototaxis'											
1	6·0	1·0	1·0	5·0	7·0	0·0	2·0	6·0	7·0	7·0	1·0	4·5
2	6·0	4·5	3·0	6·0	7·0	5·0	3·0	7·0	7·0	5·0	5·0	5·0
2	7·0	2·0	1·0	4·5	6·5	6·5	3·0	5·0	5·5	4·5	0·0	3·0
4	7·0	1·0	1·5	7·0	7·0	5·0	0·0	4·5	4·0	4·0	1·5	4·0
Mean	3·97				4·66				4·25			

DISCUSSION

Investigations into the functions of the ocelli have been made by many previous workers. Parry (1947) remarks on the irregular distribution of ocelli among insects, whilst Kalmus (1945) notes a correlation between the presence or absence of wings and ocelli in the systematic insect groups. From a consideration of the optical powers Kolbe (1893), Hesse (1908) and Link (1909*a*, *b*) suggest that the ocelli are used for the perception of distant objects, whilst Müller (1826), Lubbock (1889) and Lowne (1870) conclude that the ocelli are capable of perceiving only near objects. Von Buddenbrock (1937) supports both the above views. It has been claimed by Demoll & Scheuring (1912) that the ocelli function as distance assessors, whilst Lowne attributes to the ocelli the capacity of stereoscopic vision. These views assume that the ocelli are capable of perceiving form, a property which has been disproved by Homann (1924), Wolsky (1930) and later by Parry (1947). Other workers, viz.

Bozler (1926), Parry (1947) and Von Buddenbrock (1937), have concluded that the ocelli stimulate the compound eyes thereby serving a photokinetic function. That the ocelli play a part in phototaxis has been suggested by Müller (1931) and Friederichs (1931), Kalmus and Von Buddenbrock, but that the ocelli appear to play no part in immobile orientation (photo-akinesis) has been demonstrated by Volkonsky (1939). Lowne (1878), Homann (1924), Wolsky (1933), Parry (1947) and Lubbock (1889) suggest that the ocelli possibly perceive light intensity or quick changes in light intensity.

The present work has shown that the efficiency of phototactic reactions mediated by the compound eyes is under certain conditions significantly reduced when the ocelli are occluded. In *Calliphora* their influence is manifest only under conditions of high light intensity when the less efficient response appears to be mediated by the antero-lateral ommatidia. In *Locusta* their influence is manifest only when the sensitivity of the compound eyes is reduced by light-adaptation, and when orientation depends on the stimulation of the anterior part of the eye. In both species the experimentally observed behaviour may be explained by the following theory.

Orientation of normal *Calliphora* is such that there is equal stimulation of the anterior regions of both compound eyes, but on the occlusion of the ocelli this arrangement is upset. It is suggested that ocellar occlusion results in a decrease in the sensitivity of the ommatidia of the anterior region of the compound eye, that they are then unable to function together and that orientation is mediated by the antero-lateral ommatidia. By this process the decrease in sensitivity of the eye is made good by less direct responses in which a greater number of ommatidia become stimulated. This theory is substantiated by the results obtained with *Locusta*, for a decrease in the efficiency of response of this species is evident only when normal orientation is mediated by the anterior regions of the compound eyes, and not when the response is effected by the lateral ommatidia of one eye. It is possible that the ocelli function in co-ordinating the responses mediated by the contra- and ipsi-lateral ommatidia, described by Mast (1923). It must be pointed out that the effect of occluding the ocelli of *Locusta* is manifest only when the sensitivity of the compound eye is reduced by light-adaptation. Under these conditions the effect of occlusion may be more evident than when orientation is mediated by highly sensitive compound eyes. Also, in *Calliphora* the influence of the ocelli is evident only when stimulated by a high light intensity. It is suggested that the degree to which the ocelli influence the response depends on the intensity of stimulation and the state of sensitivity of the compound eyes.

To conclude this discussion on the contribution of the ocelli to phototaxis, one may consider the efficient phototactic orientation of normal insects to be the result of directional responses mediated by the compound eyes, the efficiency of movement being maintained by the ocelli which correct directional deviations. The suggestion that the absence of ocellar stimulation resulting from occlusion decreases the sensitivity of the anterior regions of the compound eyes does not imply that a physical change occurs in these facets, but that there is a decrease in the excitation of the nerve elements from these receptors. Such a decrease in nervous excitation

will not only be manifest in a decrease in the efficiency of directional response, but also in the speed of reaction or photokinetic response to stimulation. In immobile orientation or photo-akinesis, however, where efficiency or speed of movement is not the essential criterion of response, the stimulatory function of the ocelli to the nervous system will not be evident.

SUMMARY

1. By determining the appropriate physical constants of the ocellus it is shown that in both *Calliphora* and *Locusta* the focal plane lies much deeper than the retinal space so that the eye cannot perceive form with any accuracy.

2. The large visual fields afforded by their position on the head and the position and shape of their retinae suggest that the ocelli are efficient light-gathering units.

3. The ocelli alone are unable to mediate phototactic responses to light of the intensities employed in these experiments.

4. The efficiency of phototactic reactions mediated by the compound eyes is, under certain conditions, significantly greater when the ocelli are intact than when they are occluded.

5. In *Calliphora* this stimulatory influence of the ocelli is not manifest at a low light intensity.

6. In *Locusta* nymphs, ocellar occlusion produces an effect only when the compound eyes are light-adapted and orientation depends on light stimulating the anterior part of the eye.

7. In both species, the experimentally observed behaviour is explicable on the theory that ocellar occlusion causes a decrease in the sensitivity to light of the anterior part of the compound eye.

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THE TRANSPIRATION OF TERRESTRIAL ISOPODS

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INTRODUCTION

The transpiration of terrestrial arthropods has been the subject of some attention during recent years. It is evident from the work of Ramsay (1935*b*), Wigglesworth (1945) and Beament (1945) that in insects the effective barrier to evaporation is a thin layer of epicuticular lipoids. In view of the similarity between crustacean and insect cuticles (Pryor, 1940; Dennell, 1947) it would be of interest to determine whether isopod permeability is restricted by similar means.

MATERIALS AND METHODS

Representatives from three different genera were studied, *Oniscus asellus* Linné, *Porcellio dilatatus* Brandt and *Armadillidium vulgare* Latreille. Most of the experiments were carried out with *Oniscus*, and results will refer to this animal unless otherwise specified.

Transpiration rates were determined by suspending animals singly in a desiccation chamber. This was maintained at constant temperature, and relative humidities were controlled with potassium hydroxide solutions (Buxton & Mellanby, 1934). Animals were weighed at regular intervals on a 200 mg. torsion balance. The loss of solid material during the course of an experiment was shown to be negligible compared with the loss of water. Transpiration rates could thus be calculated on the basis of recorded weight loss.

Ramsay (1935*a*) has pointed out that certain sources of error may attend experiments carried out in still air. Tests were made to estimate the magnitude of such errors by comparing transpiration rates obtained in the desiccation chamber with values obtained in a current of air. Since no significant difference could be established it appears that the present results are not critically affected by these errors. Nevertheless, it would be useful to have some standard with which experimental results could be compared. The rate of evaporation from a free water surface was accordingly measured (see Table 1).

The relation between surface area and weight was determined so that transpiration rates could be expressed in terms of permeability.

RESULTS

(1) *The transpiration of isopods*

To establish the general level of permeability in woodlice the transpiration of *Oniscus*, *Porcellio* and *Armadillidium* was determined under different conditions of saturation deficiency. The loss of water from isopods takes place in a very charac-

teristic manner, as shown in Fig. 1, where a few typical transpiration curves are plotted.

There is a marked fall in the rate of water loss during exposure to unsaturated air. This decrease is most rapid during the early stages of desiccation; but even after

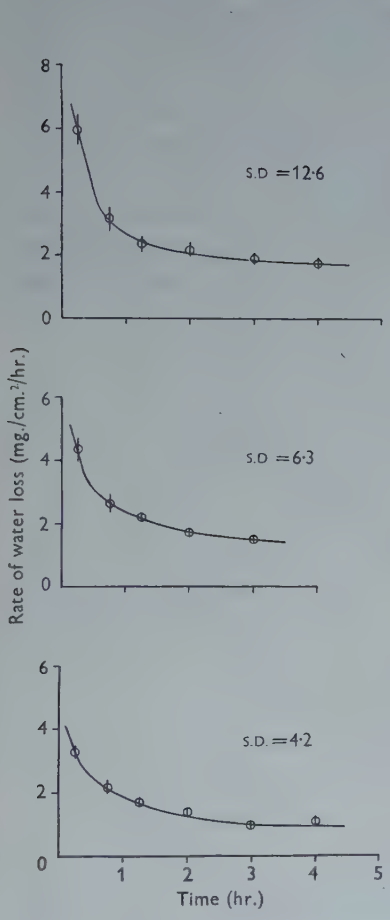

Fig. 1.

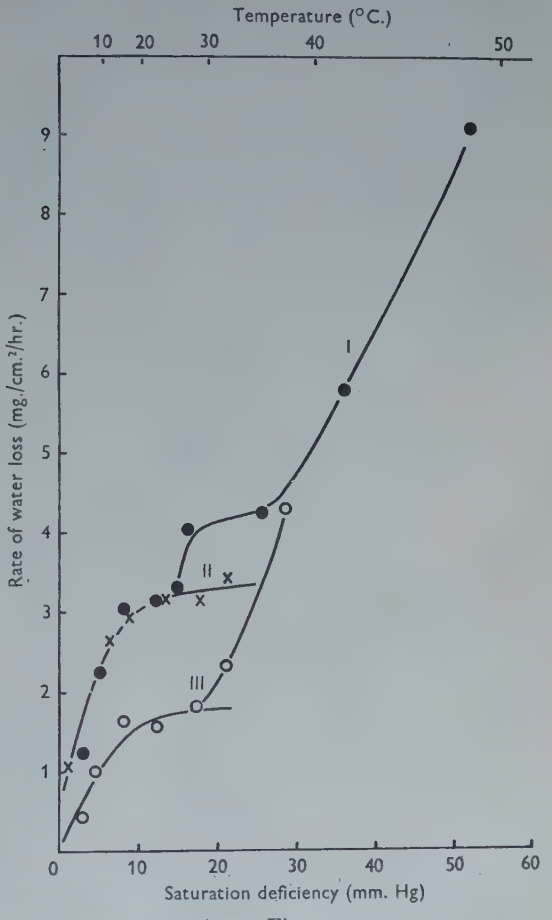

Fig. 2.

Fig. 1. The rate of water loss during exposure to different saturation deficiencies. Lines have been drawn to the fiducial limits of the means ($P=0{\cdot}05$).

Fig. 2. The effect of temperature on the rate of water loss of woodlice. I: (●), *Oniscus* exposed to different temperatures at constant relative humidity (41 %). II: (×), *Oniscus* exposed to different relative humidities at constant temperature (23° C.). III: (○), *Porcellio* exposed to different temperatures at constant relative humidity (41 %).

2–3 hr. the decline is appreciable, and it continues after the death of the animal. The phenomenon has been demonstrated for all three species over a wide range of saturation deficiencies. It seems to be a fundamental characteristic of transpiration in terrestrial isopods.

Another constant feature of isopod transpiration is illustrated in Fig. 2 (curve II),

where the rate of water loss is plotted against saturation deficiency at constant temperature (see also Table 2). Over the lower part of the range the rate of transpiration increases rapidly, but at higher saturation deficiencies the rate of increase becomes smaller and smaller and the curve tends to flatten out. In other words, the rate of water loss per unit of saturation deficiency is not constant but decreases with increasing saturation deficiency. It will be shown that this departure from the expected rectilinear relation is a function of the decrease in the rate of water loss with time.

For comparison with other arthropod groups the transpiration rates of isopods were calculated from the water loss during the third hour of desiccation. It will be shown that the rate of water loss during initial stages of exposure does not give a true reflexion of cuticular permeability. The figures for insects and ticks were obtained from the publications of Wigglesworth (1945) and Lees (1947). Loss of water from a free water surface is included in Table 1 for comparison with transpiration values.

Table 1. *The transpiration rates of some arthropods*

Temp. (° C.)	Saturation deficiency (mm. Hg)	Animal	Rate of water loss (mg./cm.²/hr./mm. Hg)
		Insects:	
20	17·5	*Tenebrio*	0·013
30	31·8	*Agriotes*	0·028
20	17·5	*Bibio*	0·046
20	17·5	*Pieris*	0·069
		Ticks:	
57	140	*Ornithodorus*	0·006
29	30·1	*Ixodes*	0·043
		Isopods:	
23	12·5	*Armadillidium*	0·056
23	12·5	*Porcellio*	0·088
23	12·5	*Oniscus*	0·176
23	12·5	Free water surface	6·16

The level of permeability is approximately the same in these different groups of arthropods. The transpiration rates of woodlice are somewhat greater than those of most adult insects and ticks. But this difference is negligible compared with the enormous difference between the water loss from isopods and that from a free surface of water. The cuticle of terrestrial Crustacea thus constitutes a highly efficient barrier to the penetration of water, only slightly inferior to the cuticles of insects and ticks in this respect.

(2) *Cuticular lipoids*

(i) *The effect of temperature on transpiration*

The low permeability of isopod cuticles suggests that lipoids may form an important constituent of the integument. In order to test this possibility the effect of temperature on the rate of transpiration was determined. The results for *Oniscus* are shown in Fig. 2 (curve I).

There is very good agreement between transpiration rates determined at constant temperature (×) and at rising temperatures (●) up to 25° C. But above this temperature there is an abrupt break in the curve indicating that an increase in permeability has taken place. Transpiration levels off to a second plateau and a second transition occurs above 35° C., after which the curve rises more steeply to high rates of water loss.

Fig. 2 shows the transpiration during the second half hour of exposure. Numerical data are given in Table 2, and it is seen that similar breaks occur during the first and third half hours.

Table 2. *The effect of humidity and temperature on the transpiration rate of* Oniscus asellus *and* Porcellio dilatatus

I. *Oniscus*

(*a*) Constant temperature (23° C.)

Saturation deficiency mm. Hg	Time (min.)		
	0–30	30–60	60–90
	Rate of water loss (mg./cm.²/hr.)		
1·1	1·12 ± 0·04	1·08 ± 0·06	1·06 ± 0·08
4·2	3·28 ± 0·11	2·16 ± 0·13	1·70 ± 0·09
6·3	4·37 ± 0·19	2·65 ± 0·15	2·21 ± 0·10
8·4	5·43 ± 0·21	3·04 ± 0·16	2·14 ± 0·11
12·6	5·94 ± 0·24	3·16 ± 0·19	2·34 ± 0·13
17·8	5·96 ± 0·21	3·16 ± 0·11	3·14 ± 0·11
21·1	6·08 ± 0·18	3·46 ± 0·11	3·04 ± 0·05

(*b*) Constant relative humidity (41 %)

Temp. (° C.)	Saturation deficiency mm. Hg	Time (min.)		
		0–30	30–60	60–90
		Rate of water loss (mg./cm.²/hr.)		
1·5	3·1	2·16 ± 0·17	1·24 ± 0·08	1·09 ± 0·10
9·5	5·3	3·88 ± 0·10	2·26 ± 0·18	1·64 ± 0·22
16·0	8·1	5·43 ± 0·21	3·04 ± 0·25	2·14 ± 0·19
23·0	12·3	5·94 ± 0·24	3·16 ± 0·19	2·34 ± 0·13
26·2	15·0	6·67 ± 0·43	3·32 ± 0·20	2·94 ± 0·07
27·5	16·3	7·12 ± 0·40	4·07 ± 0·39	3·22 ± 0·21
35·5	25·6	7·63 ± 0·33	4·26 ± 0·21	3·65 ± 0·15
42·0	36·3	8·10 ± 0·19	5·80 ± 0·23	5·52 ± 0·18
49·0	51·9	11·40 ± 0·41	9·10 ± 0·40	8·30 ± 0·36

II. *Porcellio*

Constant relative humidity (41 %)

Temp. (° C.)	Saturation deficiency mm. Hg	Time (min.)		
		0–30	30–60	60–90
		Rate of water loss (mg./cm.²/hr.)		
1·5	3·1	0·89 ± 0·09	0·42 ± 0·09	0·51 ± 0·10
7·2	4·6	1·39 ± 0·21	1·00 ± 0·18	0·38 ± 0·20
16·0	8·1	3·46 ± 0·15	1·63 ± 0·13	1·30 ± 0·12
23·1	12·5	4·33 ± 0·28	1·56 ± 0·19	1·25 ± 0·06
28·7	17·5	4·81 ± 0·24	1·82 ± 0·12	1·48 ± 0·13
32·0	21·1	6·30 ± 0·21	2·31 ± 0·11	1·78 ± 0·08
37·5	28·6	8·98 ± 0·37	4·32 ± 0·27	2·75 ± 0·15

The occurrence of two critical temperatures can be shown by subjecting a group of animals to progressively rising temperatures. As shown in Table 3 the rate of water loss decreases during the course of desiccation in the same way as for animals at constant temperature (see Fig. 1). But when the temperature is raised above 26° C. there is a rapid increase in transpiration rate, indicating that a change in cuticular permeability has taken place; the rate falls again as the temperature is raised further until at temperatures above 38° C. a second transition point is reached and the rate of water loss increases progressively.

Table 3. *The effect of a progressive rise in temperature on the rate of transpiration of* Oniscus asellus

(Relative humidity = 60%.)

Time (min.)	Temp. (° C.)	Rate of water loss (mg./cm.²/hr.)
0–30	17	4·73 ± 0·14
30–60	21	3·18 ± 0·16
60–90	24	2·82 ± 0·12
90–120	26	2·73 ± 0·08
120–150	31	3·10 ± 0·10
150–180	35	2·82 ± 0·10
180–210	38	2·64 ± 0·12
210–240	41	3·00 ± 0·18
240–270	43	3·37 ± 0·12
270–300	45	4·08 ± 0·16

To establish that the transition points are based on some passive property of the cuticle, experiments were carried out with groups of dead animals. These were killed by exposure to ammonia vapour before transpiration was determined. The humidity to which each group was exposed was chosen with reference to the temperature so that saturation deficiency was the same in all cases. Differences in the rate of water loss would be due to changes in cuticular permeability since evaporating power was constant. The results are shown in Table 4.

Table 4. *The effect of temperature on the rate of transpiration of* Oniscus *at constant saturation deficiency*

Temp. (° C.)	R.H. (%)	Saturation deficiency mm. Hg	Time (min.)			
			0–30	30–60	60–90	90–150
			Rate of water loss (mg./cm.²/hr.)			
23	15	17·8	4·02 ± 0·16	2·26 ± 0·12	2·03 ± 0·08	1·95 ± 0·09
30	44	17·8	5·50 ± 0·32	3·14 ± 0·16	2·98 ± 0·25	3·14 ± 0·22
40	68	17·8	4·98 ± 0·28	4·05 ± 0·18	3·66 ± 0·22	4·30 ± 0·24

The rate of evaporation at 30° C. is greater than at 23° C., and the rate at 40° C. is greater than both except during the first half hour. With this exception also the differences are statistically significant and show that an increase in permeability has

occurred at the higher temperatures. It is probable that these changes in permeability correspond to the appearance of transition points in the curve of Fig. 2.

The relation between temperature and water loss has been investigated for *Armadillidium* and *Porcellio* also, both of which have critical temperatures at about 28° C. The curve for *Porcellio* is shown in Fig. 2 (III), with numerical data in Table 2.

The water loss/temperature relations presented differ considerably from those of other arthropods. The transition point of the isopod curve represents a change from a progressively decreasing to a progressively increasing slope. In insects and ticks it represents a change from a negligible to a very steep slope (Wigglesworth, 1945; Lees, 1947). But there can be little doubt that the underlying mechanism of these permeability changes is the same in the two cases. The critical temperature of the isopod cuticle probably reflects a change in the state of cuticular lipoids.

(ii) *The extraction of lipoids*

An attempt was made to isolate the lipoids of *Oniscus* cuticle by extraction with fat solvents. Cast skins were used as it was difficult to prepare fresh cuticle free from adhering tissue fragments.

A number of moulted cuticles were collected and washed with water. They were dried and treated with boiling chloroform for 6 hr. under a reflux condenser. When the solvent was allowed to evaporate a small fatty residue was left at the bottom of the container. Under the microscope this residue was seen to consist of two distinct components; one formed a homogeneous matrix in which crystals of the other were embedded. When observed with polarized light the homogeneous ground substance showed a faint birefringence, while the crystalline fraction stood out brilliantly against this background.

It was not possible to stain this material effectively with the usual fat stains. Even at high temperatures the Sudan dyes were only sparsely soluble. But the extract was very effective in reducing osmium tetroxide which turned intensely black after a short period of exposure.

In an attempt to establish melting-points for the extracted lipoids advantage was taken of their birefringence. A small sample was sealed between two cover-slips, and these were placed in a flat-sided glass tube, through which water of known temperature could be circulated. The lipoids could be viewed in polarized light with a low-power objective, and a distinction could be made between the two components of the system.

The slight birefringence of the non-crystalline fraction faded at about 23° C. The lipoid crystals showed intense birefringence up to a temperature of 36° C.; above 37° C. the double refraction faded, and the crystals ceased to exist as such.

There is a fairly close correspondence between the critical temperature of the transpiration curves and the temperature at which melting of the two components occurs in the isolated lipoids. It seems probable that the lipoids which have been described in this section constitute the material bases of the permeability changes recorded above.

(iii) *The distribution of lipoids in the cuticle*

Since the isolated lipoids are capable of reducing osmium tetroxide it should be possible to demonstrate their presence in the cuticle by means of this reagent.

Pieces of fresh cuticle were immersed in a solution of osmium tetroxide, and sections were cut on the freezing microtome. The results showed that both epicuticle and endocuticle had caused intense reduction of osmium tetroxide, while the exocuticle showed no signs of staining.

If the material is extracted with boiling chloroform before treatment with osmium tetroxide, the reducing capacity of the endocuticle is greatly decreased, but it is not completely suppressed. A completely negative reaction is obtained only if the cuticle is decalcified with weak acid prior to extraction with chloroform.

The results show that materials occur in the endocuticle which are capable of causing the reduction of osmium tetroxide, and these materials are largely removed by extraction with fat solvents. They can undoubtedly be identified with the lipoids previously isolated by means of chloroform extraction.

The reduction which occurs in the epicuticle may be due to the epicuticular fats described by Dennell (1947); or to chemically combined lipoid. But some experiments, done with newly moulted animals, indicate that the epicuticle is not responsible for the low permeability of the integument.

If animals are tested soon after completion of a moult the rate of transpiration is found to be more than twice as high as that of normal controls. Drach & Lafon (1942) have shown that when the old cuticle is shed the epi- and exocuticles of the new integument are complete, but deeper layers have not yet been elaborated. Thus, in the absence of endocuticular layers, the integument offers comparatively little resistance to the diffusion of water.

The conclusion that a deep-seated lipoid impregnation constitutes the limiting barrier is confirmed by determining the effect of inert dusts. Even severe abrasion with dusts has no significant effect on the rate of transpiration. If the impermeable layers were superficial, as in insects, this treatment would result in a very substantial increase in the rate of water loss (Beament, 1945).

(3) *The decrease in transpiration rate with time*

The deep-seated nature of impermeable layers will in some part account for the characteristic form of isopod transpiration curves. On exposure to unsaturated air a gradient of vapour pressure will be set up across the cuticle, and this will entail a rapid loss of water from the relatively permeable outer layers of the integument. The rate of water loss will decrease rapidly as the gradient becomes established, and until this happens transpiration will not give a true indication of cuticular permeability.

But after the rapid decline there follows a steady decrease in the rate of transpiration which cannot be accounted for on this basis. This secondary decrease would suggest that a change is taking place in the permeability of the cuticle, and some experiments were carried out to determine the nature and mechanism of this change.

(i) *The relation between water content and permeability*

A decrease in permeability could be due to a progressive drying up of the outer layers of the cuticle. King (1945) and Gluckauf (1944), working with keratin membranes, have shown that the rate of diffusion of water vapour is dependent on the degree of hydration of the keratin. A similar phenomenon has been shown to be a general property of hygroscopic materials (Babbitt, 1940). But preliminary experiments with *Oniscus* suggested that permeability was a function of the extent of desiccation, rather than of the degree of hydration of the cuticle. To explore this possibility the effect of desiccation on water loss was determined using animals which had been brought into equilibrium with saturated air, so that their cuticles were fully hydrated.

The animals were exposed to dry air for different lengths of time. They were then left in 100 % R.H. for 5–6 hr. during which time a certain amount of water was absorbed. This absorption was presumably associated with the disappearance of a vapour pressure gradient across the cuticle. Equalization of vapour pressures within the cuticle would be brought about by diffusion of water into regions of low from regions of high vapour pressure, that is, by diffusion into the outer layers both from the blood and from the saturated atmosphere. The proportion of water deriving from these possible sources would be determined in part by the permeability of the inner layers of the cuticle, and in part by the hygroscopic properties of the cuticular substance. When the permeability is low a considerable amount of water will be taken up from the atmosphere. This would appear to be the case with *Oniscus*. The uptake is rapid to begin with but declines sharply during the first half hour, being complete after about an hour. Thus after 5–6 hr. in saturated air the cuticle may be assumed to have regained its maximal degree of hydration.

The animals were then exposed to some test humidity, and the rate of transpiration during the first half hour was determined. Water content was used as a measure of the extent of previous desiccation, and the transpiration rate of each animal was expressed as a percentage of mean control rates for undesiccated animals. The results of all experiments are summarized in Fig. 3, and it is clear that the rate of transpiration is a function of the extent of previous desiccation. The lower the water content of the animal the lower is the rate of evaporation.

The regression of transpiration rate on water content is of the right order of magnitude to account for the observed decrease in transpiration rate with time. For example, during exposure to 0 % R.H. for $1\frac{1}{2}$ hr. the water content of *Oniscus* drops from 66 to 60 %; on the basis of Fig. 3 the water loss should decrease to 47 % of its original rate. The observed decrease is from 7·0 to 3·1 mg./cm.^{2}/hr., that is to 44 % of the original rate. In spite of this close correspondence the possibility cannot be excluded that a decrease in permeability brought about by drying out of the cuticle may contribute to the fall in transpiration rate with time.

These experiments show that a decrease in permeability occurs which is independent of cuticular hydration. It seemed possible that this decrease could depend on changes in the concentration of fluids which bathe the transpiring

surfaces, and it was necessary to establish whether such changes occurred during desiccation.

ii) *The effect of desiccation on the salt concentration of body fluids*

The apparatus for dealing with this problem was put at my disposal through the kindness of Dr J. A. Ramsay. The method depends on determination of the freezing-point of very small quantities of fluid and has been described elsewhere (Ramsay, 1949).

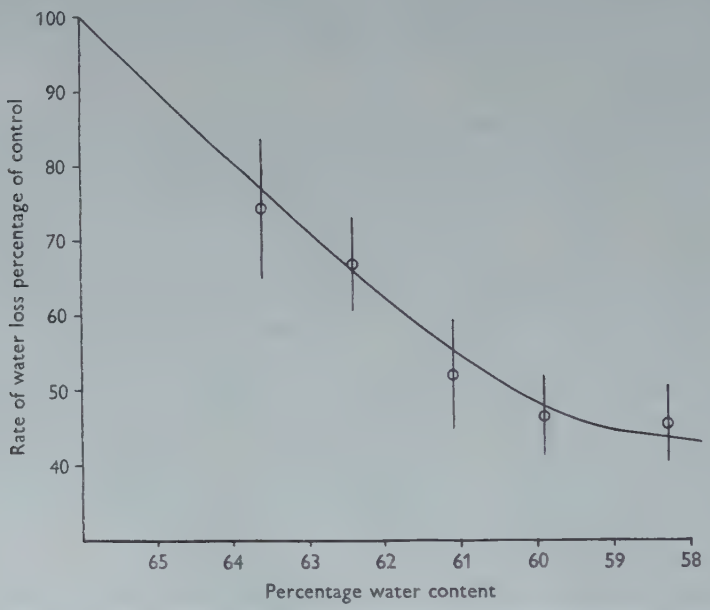

Fig. 3. The effect of previous desiccation on the rate of water loss. The data have been grouped for water content and are plotted against the mean value for each of the five groups. Lines have been drawn to the fiducial limits of the means ($P=0{\cdot}05$). The correlation coefficient calculated from the ungrouped data is 0·55 ($P=0{\cdot}01$).

By the use of a simple capillary pipette it was possible to withdraw several samples of blood from the dorsal blood sinus of *Oniscus* without apparent deleterious effect. The freezing-points of successive samples from animals kept on moist filter-paper showed no significant variation.

Table 5 shows the changes in blood concentration during desiccation. Animals were weighed before and after sampling, and the blood concentration could thus be expressed as a function of water content.

It is clear that during desiccation there is a marked increase in the salt concentration of body fluids. The concentration of blood from undesiccated animals is 1·59%; values calculated on the assumption that the water lost has been withdrawn from a 1·59% salt solution of volume equal to the volume of water originally present in the animal agree well with experimental figures. This correspondence shows the absence of an active regulation of total blood concentration.

The concentration of osmotically active substances in the blood increases as the animal loses water; there is a simultaneous decrease in cuticular permeability. It seemed possible that these two phenomena might be causally related, and that the increased salt concentration of the blood in some way modifies the structure of the cuticle so as to cause a decrease in permeability. The crustacean cuticle contains considerable quantities of protein (Lafon, 1943, 1948), and an interaction between these and their ionic environment might produce effects of this kind.

Table 5. *Changes in the blood concentration of* Oniscus *during desiccation*

Mean water content (%)	Blood concentration (% NaCl)	Blood concentration calculated, (see text)	No. of determinations N
66·0	1·59	—	13
64·2	1·73	1·74	5
62·6	1·91	1·88	5
60·0	2·00	2·09	11

(iii) *The effect of salt concentration on the structure of the cuticle*

(*a*) *Macroscopic changes.* Changes in the permeability of the cuticle would presumably be associated with changes in submicroscopic structure. It seemed possible that such changes might be reflected in corresponding changes of macroscopic dimension. To investigate this possibility a perfusion cell was made which enabled fresh sections of cuticle, cut on the freezing microtome, to be studied continuously under a high-power objective. The solutions bathing the section could be changed rapidly without disturbing the preparation. Outline drawings of a projected image of the section were made, and it was possible to establish changes amounting to 0·5–1·0% of the thickness of the cuticle with certainty.

Cuticle thickness was measured at a series of reference points and a mean value was calculated; this was expressed as a percentage of the thickness in 0·275N sodium chloride, the concentration of the blood of undesiccated animals.

The results are shown in Fig. 4*a*. It is clear that sodium chloride concentration has a marked effect on the thickness of the cuticle. The changes indicate that as the salt concentration is raised from 0·100 to 0·275 N the cuticle swells; with a further increase in ionic strength shrinkage occurs up to concentrations of 2·000 N.

(*b*) *Submicroscopic changes.* Another way in which changes in cuticular structure may be demonstrated depends on the sorption of water vapour. If a dry piece of cuticle is exposed to saturated air a certain amount of water vapour will be adsorbed. The actual quantity will depend on the nature, the extent and the disposition of adsorbing surfaces in the cuticle. Any change in structure may be reflected in a difference in the amount of water vapour which is taken up.

To study this possibility pieces of moulted cuticle were immersed in solutions of different ionic strengths. In order to facilitate penetration exposure was carried out at 60° C. After 2 hr. the cuticle was rapidly washed free of adhering salt solution by a 1 min. immersion in cold tap water. It was blotted and dried to constant

weight. On subsequent exposure to 100% relative humidity the amount of water vapour adsorbed at equilibrium was determined. The results are expressed as a percentage of the dry weight (% regain) and are summarized in Fig. 4*b*.

The relation between salt concentration and percentage regain is similar to that shown for cuticle thickness in Fig. 4*a*. The high adsorption after exposure to

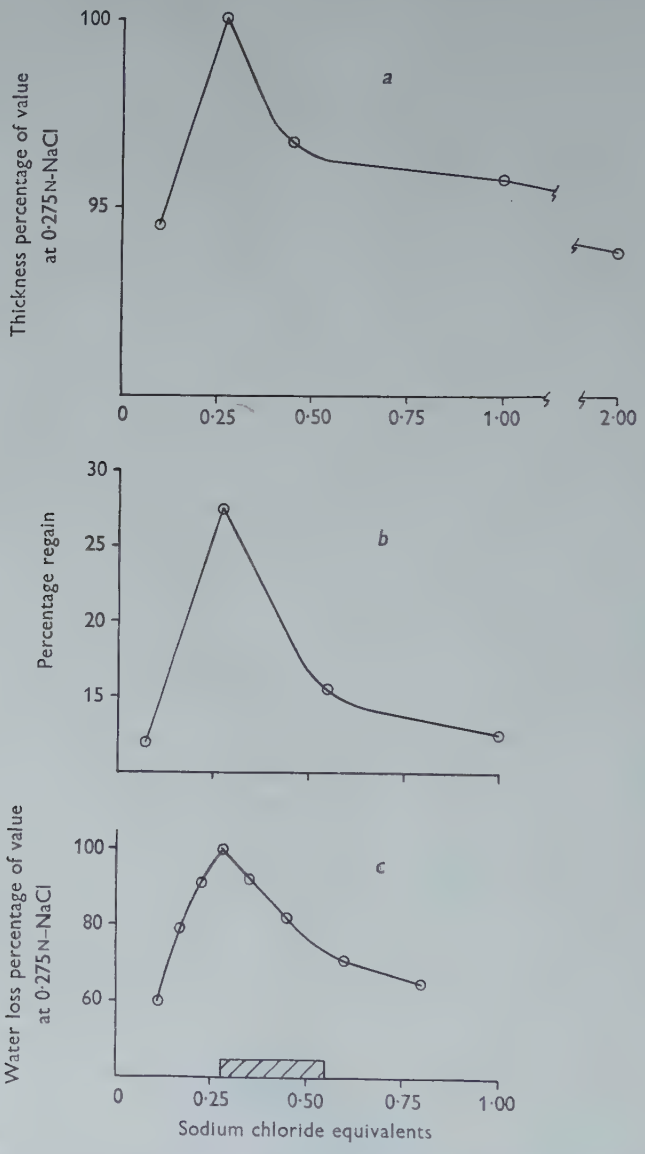

Fig. 4. The effect of salt concentration on cuticular structure. (*a*) Effect on the thickness of the cuticle. (*b*) Effect on the adsorption of water by moulted cuticle. (*c*) Effect on permeability of perfused cuticle. The range of blood concentrations observed during desiccation of *Oniscus* is marked on the abscissa.

0·275 N sodium chloride is probably an expression of a comparatively open submicroscopic structure. The surface area available for adsorption would be relatively large and so would the capacity of any capillary spaces which may exist in the fine structure of the membrane (Barrer, 1941; Bull, 1944; Sponsler, Bath & Ellis, 1940). With higher or lower salt concentrations the cuticle becomes more compact, and there is a corresponding decrease in the water-holding capacity.

(*c*) *Permeability changes.* To see whether these changes in the structure of the cuticle would cause corresponding changes in cuticular permeability it was necessary to study the rate of water loss through isolated cuticle which could be bathed with solutions of known concentration. A number of attempts were made to find a method of dealing with this problem. The calcareous nature of the cuticle proved the main obstacle as it rendered the integument very liable to cracking.

The method finally adopted was to remove head and first body segment which come off together with the gut and digestive glands. The body cavity was scraped with a fine metal hook and washed with sodium chloride solution to remove as much tissue as possible. The preparation was then mounted on two fine glass capillaries inserted through the second body segment, and the body cavity was sealed off at anterior and posterior ends with cellulose paint.

The capillary tubes were connected to a perfusion set, so arranged that hydrostatic pressure and rate of flow of perfusing liquid could be accurately controlled. The solution flowing through the preparation could be rapidly changed by switching to one or other of two reservoirs.

The preparation could be inserted into a desiccation chamber containing a small tray of calcium chloride suspended on a torsion balance, so that the amount of water passing through the cuticle could be measured at short intervals. The air inside the chamber was kept in circulation by a small fan operated from the outside by a rotating magnet. The whole was enclosed in a water-jacket through which water from heating coils immersed in a water-bath could be circulated. A thermoregulator in the desiccation chamber controlled the temperature of the water-bath, and by this means the temperature inside the chamber was maintained at $22{\cdot}0 \pm 0{\cdot}5^{\circ}$ C.

With this apparatus a measure of permeability could be obtained in 30 min., so that a number of determinations could be made in a single day. This made it possible to check the constancy of the preparation by determining the rate of water loss under standard conditions at the beginning and end of each series.

A serious drawback to the method is that determination of transpiration rates is empirical, since it is impossible to estimate the extent of the cuticle which is actually being perfused. This means that observations have only relative value; but if results are expressed with reference to transpiration under a standard set of conditions the data obtained with different preparations will be comparable.

In the first series of experiments solutions containing a variety of ions were employed. But it was found that the rate of water loss was the same whether a balanced solution was used, or a solution of pure sodium chloride, provided ionic concentration and pH were the same. So for the sake of simplicity pure sodium chloride was subsequently used. The solutions were buffered with Na_2HPO_4 and

the pH adjusted to 7·5 with sodium hydroxide. This value corresponds with the pH of *Oniscus* blood as determined roughly with indicators (Gorvett, 1950, records a higher value for isopods).

The results of all experiments on the effect of salt concentration on permeability are shown in Fig. 4*c*. With distilled water (and possibly at extremely low salt concentrations) an irreversible change in cuticular permeability takes place, and the integument becomes freely permeable to water.* At a salt concentration of 0·110 N cuticular permeability is very low; it rises with increasing concentration to a peak at 0·275 N; subsequently there is a sharp drop and the curve tends to level off above concentrations of 0·600 N.

The relation was tested at different values of pH. The shape of the curve was found to be independent of the reaction of the perfusing medium, but the level of permeability varied greatly (see below).

The curve is very similar to those showing the effect of salt concentration on cuticle thickness and adsorption; there can be little doubt that the three phenomena represent different aspects of an interaction between cuticular elements and ionic medium. The nature of this interaction cannot be discussed in detail. The curves show certain similarities to the solubility curves of protein in solutions of different salt content (Cohn, 1932). Such resemblances indicate that salting-in and salting-out of endocuticular proteins may lie at the base of the effects described. But a detailed study of the problem lies outside the scope of the present investigation.

The data presented suggest that changes in the salt concentration of the body fluids of woodlice are likely to produce changes in the permeability of the integument. Over the range of concentrations observed with *Oniscus* (marked on the abscissa in Fig. 4*c*) there is a progressive decrease in cuticular permeability with increasing sodium chloride concentration. It is probable that the decrease in permeability which has been described for living animals may be an expression of this phenomenon. During exposure to dry air the water content of the animal decreases; this causes a concentration of body fluids. The consequent shrinkage of the endocuticle brings about a closer packing of the impregnating fat molecules and a fall in permeability.

That proteins of the endocuticle in particular are involved in these effects is indicated by the effect of pH on the permeability and on cuticle thickness. Both are at a minimum when the pH is about 4; this value corresponds closely with the isoelectric point of decapod endocuticle as determined by Yonge (1932) and confirmed for *Oniscus* during the present investigations.† A correspondence between the pH of isoelectric point and the pH of minimal swelling is shown by most protein gels (Jordan Lloyd & Shore, 1938).

* Richards & Korda (1948), working with the electron microscope show that distilled water causes the appearance of a coarse fibrous network in the cuticle of arthropods; there appears to have been some sort of precipitation of constituent molecules.

† The isoelectric point of the epicuticle is above pH 5 (Yonge, 1932; Dennell, 1946).

CONCLUSION

On the basis of the foregoing results it is possible to offer an explanation of the fall in the rate of transpiration with time which is so characteristic a feature of desiccation in isopods. On exposure to dry air a gradient of vapour pressure is established across the cuticle, and this entails a rapid loss of water from exo- and epicuticles. The initial stages of desiccation are therefore characterized by very high rates of transpiration, which fall rapidly as the gradient approaches stable values. Simultaneously with the loss of water from the outer layers there is a diffusion of water from the blood to the surface along the gradient of vapour pressure. The consequent loss of water from the blood causes a progressive shrinkage of the cuticle with a decrease in permeability, and hence there is a steady decrease in the rate of water loss from the animal as a whole. It is possible that a decrease in cuticular hydration may contribute to this compacting of the cuticle.

Attention has been drawn to the apparent decrease in the permeability with increasing saturation deficiency (see Fig. 2; noted also by Edney, 1951). This anomaly finds interpretation in the light of later findings. It has been shown that cuticular permeability is proportional to water content (Fig. 3). For a given length of exposure animals which have been in high saturation deficiencies will have lost more water than animals which have been in low saturation deficiencies; their water content will be less and so will their permeability. Since transpiration rates at different saturation deficiencies are compared after equal durations of desiccation there will be an apparent decrease in permeability in drier air. Unless rates of water loss are compared not after equal duration, but after equal extent of desiccation, a rectilinear relation between transpiration rate and saturation deficiency cannot be expected.

DISCUSSION

A close similarity has been shown to exist between crustacean and insect cuticles as regards their general structure (Dennell, 1947). This similarity does not extend to the means by which the integument is waterproofed in the two groups. In insects the cuticle is rendered impermeable by a very thin layer of lipoid situated near the surface of the cuticle. In terrestrial isopods the diffusion of water is limited by a lipoid impregnation of endocuticular layers separated from the surface by exo- and epicuticles. Although these layers are much more permeable than is the endocuticle they may play an important role in the mechanism of waterproofing by preventing vapour pressure gradients from exerting their full force across the lipoid barrier.

Differences in the organization of lipoid are probably responsible for differences as regards the water loss/temperature relations of arthropod groups. Breaks in the isopod curves are of a gradual nature, while in insects and ticks the transition points are usually sharply defined. An exception to this general rule has been reported by Beament (1949); transpiration through the secondary wax layer of *Rhodnius* egg shell is characterized by a gradual transition point; but the lipoids concerned are

disposed in the form of an impregnation of the fertilization membrane rather than as a discrete layer.

Another difference between isopods on the one hand, and insects and ticks on the other, is that in isopods the transpiration curve above the critical temperature is not nearly so steep as in the other groups (see Edney, 1951, fig. 3, where *Blatella* is plotted on the same graph as a group of isopods). The low rate of increase of transpiration with temperature for woodlice is probably correlated with the presence of impregnating rather than free lipoids. The thermal agitation of lipoid molecules above critical temperatures would be restricted by the non-lipoid structural framework, and the membrane would possess appreciable waterproofing capacity even at high temperatures. With insects and ticks, disorganization of the oriented lipoid is free to increase progressively as the temperature is raised, until the rate of transpiration approximates to evaporation from a free water surface.

This interpretation is borne out by the work of Lees & Beament (1948), who found that in the transpiration curve for the egg of *Ornithodorus moubata* the steepness of the slope above the critical temperature decreases greatly in the course of development; and the change is apparently associated with a gradual infiltration of lipoids, at first superficial, into the substance of the egg shell.

The egg of *Rhodnius* may provide another example of this phenomenon; the slope of the curve for eggs possessing the primary wax layer only is much steeper than that which obtains after the secondary wax impregnation has been laid down.

The presence of two distinct types of lipoid, one a low melting-point grease and the other a crystalline wax, has been demonstrated in the egg of *Ornithodorus* (Lees & Beament, 1948). The critical temperature of the egg membrane is intermediate between the melting-points of these two lipoids; this is contrary to the condition in *Oniscus* where each lipoid imposes its characteristic transition point on the water loss/temperature curve. This discrepancy may depend on a difference of molecular organization in the two cases. The mixture of lipoids in the case of the tick egg may be of an extremely intimate nature, approaching the limiting condition where molecules of the two types alternate in the oriented monolayer. Under these conditions a 'mixed' transition point, such as that observed, would result. In *Oniscus* the system may have the nature of a mosaic; multimolecular aggregates of one lipoid species may adjoin similar aggregates of the other. In this case there is reason to suppose that two separate transition points might characterize the impregnation.

Simultaneously with the present investigations a comparative study of transpiration in woodlice was in progress. A preliminary note was published by Edney (1949) and the full results have subsequently become available (Edney, 1951). Edney finds no evidence for transition points in the water loss/temperature curves, and concludes from his determination of transpiration rate that there is no efficient check to evaporation in terrestrial isopods. These conclusions are at variance with the ones reached in the present account, but there is no contradiction in experimental results. It is clear from Fig. 2 above that if determinations had been made at 10° C. intervals as in Edney's investigations, the points would fit fairly well on

a straight line. The critical temperatures only become apparent when the relative decrease in the rate of water loss with increasing saturation deficiency is taken into account, and when determinations are made at intermediate temperatures.

It has been indicated that during initial stages of desiccation the water loss derives largely from water held in the exo- and epicuticles, and its rate cannot be taken as a reflexion of cuticular permeability. If this initial water loss is included in estimates of transpiration, as in Edney's experiments, abnormally high transpiration rates are recorded, which suggest that the integument is very inadequately waterproofed.

The subsequent investigations of Edney (1953) have shown that rapid loss of water from terrestrial isopods may be of great biological importance during exposure to insolation. But it is conceivable that if such exposure were prolonged the benefit conferred by ability to cool the tissues would be offset by the danger of desiccation. The presence of a substantial exocuticle with considerable water-holding capacity, and of an endocuticle whose permeability decreases with desiccation would seem particularly well suited to provide against the possibility of overheating on the one hand, and of desiccation on the other.

The concepts arrived at during the present investigation stand in marked contrast to the conclusions reached by Yonge with regard to the relative permeability of crustacean epi- and endocuticles (Yonge, 1936, 1946). A study of the lining of the foregut in *Homarus* and of *Homarus* egg-shells suggested that permeability is controlled entirely by the epicuticle. The endocuticle played no part in regulating the diffusion of substances in and out through the integument. Objections have been raised to Yonge's experiments by Richards & Korda (1948); these authors state that purified membranes of the type employed are without biological significance for studies on permeability, since membrane structure is extensively altered by treatment. Present experiments with perfused cuticle showed that even distilled water may cause irreversible damage to the endocuticle; but the epicuticle shows a striking resistance to the action of strong chemical reagents. It seems possible that under the conditions of Yonge's experiments the endocuticle had suffered extensive damage with consequent destruction of semipermeability, while the epicuticle had remained comparatively unchanged. The permeability of the integument as a whole would then reflect the permeability of the epicuticle alone.

Heeley (1941) attributed the imperviousness of isopod cuticle largely to calcification, and this view is shared by Gorvett (1946) and Lafon (1948). The present work has shown that the waterproofing mechanism is based on a lipoid impregnation of the endocuticle. Calcium may play an important role as a component of the substrate for this impregnation, but it is not itself concerned with limiting the diffusion of water. The permeability of different species cannot be correlated with different degrees of calcification; thus the integument of *Porcellio dilatatus* is very soft compared with that of *Oniscus*, yet its permeability is much lower. And the calcification of egg-bearing females of *Oniscus* is less extensive than that of normal adults, but their transpiration rates are no different. A similar independence of cuticular hardness and permeability has been demonstrated for insects (Wigglesworth, 1948;

Eder, 1940); in this case the hardness is an expression of sclerotization rather than of calcification.

The permeability of *Oniscus* cuticle has been shown to depend on the nature of the solution in contact with its inner surface (see Fig. 4*c*). Analogous phenomena have been reported for the permeability of various natural membranes to water (Brauner & Brauner, 1943; Orru, 1939). The rate of penetration was found to be minimal at the isoelectric point of the membrane proteins, as with *Oniscus*. The permeability changes can be interpreted on the basis of an interaction between protein molecules and ionic medium, leading to changes in dispersion of the proteins and consequent alteration of membrane structure. In the case of woodlice the proteins are associated with chitin and these constituents form a substrate for the impregnation of lipoids. Shrinkage of the cuticle will lead to condensation of the ground substance, and hence to a closer packing of lipoid molecules; the resistance to aqueous diffusion will consequently increase. This system bears some resemblance to the scheme put forward by Hurst (1948) in an attempt to account for the asymmetrical permeability of *Calliphora* larval cuticle. In both cases the packing of impregnating lipoid molecules is influenced by changes in the organization of structural proteins.

SUMMARY

1. The transpiration of three species of Isopoda has been investigated; *Armadillidium* shows the highest resistance to desiccation, *Oniscus* the least, and *Porcellio* is intermediate.

2. The permeability of isopod cuticle is limited by lipoids which impregnate the endocuticle. If the temperature is raised above the lipoid melting-point a marked increase in permeability results.

3. Isopods show a characteristic decline in transpiration rate during exposure to desiccating atmospheres. The high initial rate of evaporation is due mainly to loss of water from layers of the cuticle external to the lipoid barrier.

4. There is a progressive decrease in the permeability of the integument during desiccation. This is brought about by a shrinkage of the cuticle, which leads to a closer packing of lipoid molecules. The mechanism of this structural alteration is based on an interaction between endocuticular proteins and the ionic constituents of body fluids; in the course of desiccation the blood concentration increases and there is a corresponding decrease in the dispersion of endocuticular elements.

The results presented were embodied in a thesis submitted at the University of Cambridge in 1950. I wish to acknowledge my indebtedness to Prof. J. Gray, and to Drs J. W. S. Pringle, J. W. L. Beament, A. D. Lees and J. A. Ramsay for encouragement and advice during the course of the investigation. My thanks are also due to Mr R. Jewell who prepared the diagrams for this paper.

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CUTANEOUS RESPIRATION IN WOODLICE

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INTRODUCTION

There is experimental evidence, reviewed by Mödlinger (1931) and by Edney (1954), that oxygen is absorbed by woodlice through flat, biramous abdominal appendages known as pleopods. In the more advanced species, the exopodites of either the first two or of all five pairs of pleopods each possess an internal tuft of tubules (pseudotracheae) opening to the exterior by a pore, and it is these species which, in general, are associated with drier habitats. The main purpose of the present work was to find whether cutaneous respiration (meaning respiration through the general body surface apart from the pleopods) also occurs, and if so to define its extent. The fact that the integument of woodlice is relatively permeable to water suggests that such respiration is not unlikely: the possibility has been briefly referred to by Verrier (1932) and Macagno (1938, 1939), but it has never been critically investigated.

MATERIAL AND METHODS

Four species of woodlice were used, and they were chosen to cover, so far as possible, the range of habitats and of modification of the pleopods in the British species. *Ligia oceanica* Linn. is a littoral species; it is larger than the others (up to 3 cm. long) and possesses unmodified pleopods. *Oniscus asellus* Linn. is also without pseudotracheae, but the exopodites are excavated near their outer margins to form a number of simple air spaces. *Porcellio scaber* Latr. is capable of surviving, at least for short periods, in somewhat drier regions than *Oniscus*, and possesses well-developed pseudotracheae on the exopodites of the 1st and 2nd pairs of pleopods. *Armadillidium vulgare* Latr. is the most terrestrial of all the species here studied and also possesses two pairs of pseudotracheae. These species also stand in the above order as regards rate of water transpiration through the integument (from most to least rapid) (Edney, 1951).

The procedure adopted was to compare the rates of oxygen absorption and carbon dioxide output in normal animals with those in animals whose pleopods were blocked. Two coats of an emulsion paint were used to block the pleopods. This substance is harmless to the animals (they live indefinitely if painted on the back), it flows easily over the hygrophilic surface of the pleopods, and on drying is nearly or quite impermeable to oxygen.

The permeability of this substance to oxygen was investigated in two ways. In the first method, a small glass container with a well-fitting rubber bung was half

filled with a slightly acid solution of pyrogallol. The bung possessed two holes, through one of which a glass tube from a nitrogen supply entered; the other was covered with a small piece of fine muslin. Oxygen was then removed from the solution and the air above it by bubbling nitrogen through for 1 hr. Excess alkali was then added to the pyrogallol by means of a hypodermic needle through the rubber bung. If the muslin covering the second hole was left untreated, the pyrogallol turned brown within a few minutes, but if the muslin had previously been painted with two coats of emulsion paint, the solution remained colourless for at least 12 hr. and then began to turn brown very slowly.

In the second method, reduced indigo was injected into living woodlice. When normal animals were used, the pleopods turned deep blue (the colour of oxidized indigo), but if the pleopods had been painted, and the paint allowed to dry, no blueing of these organs was observed. If the pleopods on one side only were painted, they alone remained colourless, those on the opposite side turning deep blue.

It may therefore be accepted that a layer of dried emulsion paint is sufficiently impermeable to oxygen to act as a block for the purposes of the present experiments.

Respiration was measured by means of Dixon's (1934) modification of the Barcroft manometer, at 22° C., and the results reduced to standard temperature and pressure. When oxygen uptake was being measured, 4% potassium hydroxide on Whatman's no. 40 filter-paper was used to absorb the carbon dioxide; when carbon dioxide output was being measured, the filter-paper was moistened with 0·5 ml. of 1% sulphuric acid, and the necessary correction for solubility was made. The air in the respiration flasks was therefore nearly saturated with water vapour. When respiration in dry air was being measured for comparison extra precautions had to be taken, and these will be described below.

The respiration rates obtained have been expressed in terms of surface area rather than body weight, since Ellenby (1951) found the rate of oxygen absorption in *Ligia oceanica* to be more nearly proportional to surface area than to weight. In practice, the surface area of a woodlouse is not easy to determine, and the values obtained previously (Edney, 1951) for k in the expression $S = kW^{\frac{2}{3}}$ were again employed. The error in absolute terms may be considerable, but at least it is consistent between individuals of one species. For some purposes it may be more convenient to have the results in terms of unit weight (mm.^{3}/mg./hr.), and this may readily be obtained, for one size of animal, by multiplying the present figures by a factor which varies from one species to another. These factors, which apply to animals of a size representative of those used in the present work (where very large and very small animals were excluded) are as follows: *Ligia* (0·8 g.), ×1·24; *Oniscus* (0·1 g.), ×2·86; *Porcellio* (0·1 g.), ×2·63; *Armadillidium* (0·12 g.), ×2·35.

The numbers of individuals used in each determination were: *Ligia*, 1; *Oniscus* and *Porcellio*, 10; *Armadillidium*, 10 or 5. The manometer scale, and the surface area of the animals, were such that measurements could be made to the nearest 0·005 mm.^{3}/mm.^{2}.

EXPERIMENTS

(*a*) *Respiration of normal and blocked animals in moist air*

Preliminary experiments having shown no significant difference between the respiration rates of male and female animals (provided the latter were without brood pouches) the two sexes were used indiscriminately. Each determination was carried out for 1 hr., during which readings were taken every 10 min. Twenty such determinations were made of oxygen uptake by normal animals of each of the four species. After each oxygen determination, the same animals were immediately used for carbon dioxide determination. Further experiments were made in which carbon dioxide output was measured first, followed by oxygen absorption in the same animals, and the results showed that the rates measured were in no way affected by the order in which they were obtained. Five further determinations were carried out with each species over 6 hr. periods, and these showed that both oxygen absorption and carbon dioxide excretion remained remarkably steady over this longer period.

Experiments were then carried out with animals in which the pleopods had been blocked as described above. Twenty determinations were made of the oxygen absorption in each species, and twenty more of the carbon dioxide output, but in this case the two determinations were made on different groups of animals because carbon dioxide output did not remain steady, but was always lower during the second hour than during the first.

At intervals during these determinations control experiments were run. Woodlice of the appropriate species, either normal or blocked, were killed by immersion for a minute in water at 90° C. They were then dried on filter-paper and subsequently treated in the same way as the experimental animals. No measurable oxygen consumption or carbon dioxide output was found in these controls, which therefore showed that the apparatus was working satisfactorily and that no spurious results were being caused by application of the emulsion paint used for blocking the pleopods.

The main results described above are shown in Table 1, and may now be considered in more detail. The mean rates of oxygen consumption by normal animals were (in $mm.^3/mm.^2/hr.$): *Ligia*, 0·155; *Oniscus*, 0·075; *Porcellio*, 0·084; *Armadillidium*, 0·086; so that oxygen consumption in these terms is considerably higher in *Ligia* than in the other species. If these same results are expressed in terms of body weight, the figures are (in $mm.^3/mg./hr.$): 0·192, 0·214, 0·221 and 0·202 respectively, and the difference between *Ligia* and the rest is much smaller. This suggests that when different species are compared, oxygen consumption is more nearly proportional to weight than to surface area. In earlier experiments, much variation was encountered in the oxygen consumption of animals from different sources which were presumably in different physiological conditions, and it was only by obtaining a large number of animals from one source and using them for the main experimental runs, that more consistent results were obtained. Even so, the variation, as shown by the standard error figures included in Table 1, was considerable. In addition, the factors applied for obtaining surface area from body weight are not very reliable,

so that little significance can be attached to the differences between the species, except in the case of *Ligia*, whose relatively high oxygen consumption in terms of surface area has already been mentioned.

Table 1. *The rates of respiration in mm.³/mm.²/hr.* of woodlice with normal and blocked pleopods in moist air*

(Each entry is a mean (±standard error of the mean) of twenty determinations. Calculation of R.Q. values is explained in the text.)

		Oxygen uptake	Carbon dioxide output	R.Q.
Ligia oceanica	Normal	0·155±0·0058	0·128±0·0048	0·82
	Pleopods blocked	0·081±0·0051	0·113±0·0043	1·40
Oniscus asellus	Normal	0·075±0·0030	0·064±0·0030	0·88
	Pleopods blocked	0·039±0·0024	0·052±0·0025	1·36
Porcellio scaber	Normal	0·084±0·0029	0·070±0·0028	0·85
	Pleopods blocked	0·029±0·0019	0·053±0·0031	1·69
Armadillidium vulgare	Normal	0·086±0·0033	0·077±0·0032	0·90
	Pleopods blocked	0·022±0·0018	0·036±0·0029	1·64

* For conversion of these figures to mm.³/mg./hr., see p. 257.

Ellenby (1951) obtained a mean oxygen consumption for *Ligia* weighing 0·8 g. of about 0·21 mm.³/mg./hr. at 25° C., with which the present results compare well. Other measurements of respiration in terrestrial isopods are those of Reinders (1933), who obtained a mean oxygen consumption for *Porcellio* of 0·15 mm.³/mg./hr. at 16° C., Morrison (1946): 0·35 mm.³/mg./hr. (reliability unknown) for *Oniscus* at 25° C., and Edwards (1946): 0·348 mm.³/mg./hr. (reliability unknown) for *Oniscus* at 17° C. Allowing for the difference in temperature, Reinders's figures are consistent with the present results; those of the other two authors are not very well established.

Carbon dioxide output by normal animals remained steady during the present determinations, and the respiratory quotients, calculated as means of the R.Q.'s for each pair of determinations, were reasonably consistent, varying from 0·82 (*Ligia*) to 0·90 (*Armadillidium*).

As regards the effect of blocking the pleopods, the results show a marked decrease in oxygen absorption, but it appears that all four species are nevertheless capable of absorbing some oxygen through the rest of the integument. The amount so absorbed is greatest in *Ligia* and *Oniscus* (52% of normal), less in *Porcellio* (34% of normal) and least in *Armadillidium* (26% of normal), and with the exception of *Ligia* the animals stand in the same order in this respect as they do in respect of permeability of the integument to water. The cutaneous absorption of oxygen by *Ligia* appears on this assumption to be too low, but there is no reason to expect a strict correspondence for there may well be factors other than permeability of the integument to water which limit cutaneous absorption.

As mentioned above, carbon dioxide output by animals with blocked pleopods was by no means steady. It fell more or less gradually from something like the

normal figure at the beginning of the first hour to a lower value. Experiments with blocked *Armadillidium* and *Porcellio* could not be carried out for more than 2 hr. because they began to suffer from lack of oxygen and soon died (see p. 264), but during the second hour the mean carbon dioxide output remained above the mean oxygen uptake figure, so that the R.Q. remained greater than 1. Blocked *Oniscus* and *Ligia* lived longer, but even in these species the R.Q. usually remained greater than 1 for 6 hr.; but the behaviour in this respect was very variable. The mean carbon dioxide outputs during the first hour after blocking and drying are shown in Table 1.

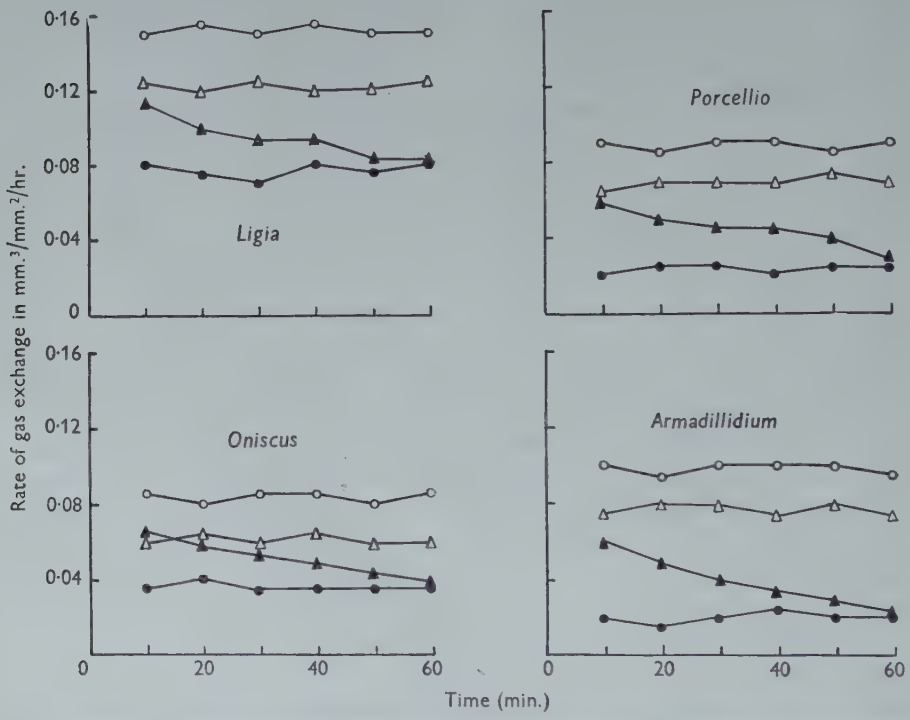

Fig. 1. The rate of oxygen uptake (circles) and carbon dioxide output (triangles) in four species of woodlice. Open symbols, normal animals; black symbols, animals with the pleopods blocked.

It must be remembered, however, that the rate of production was falling during this time, so that the R.Q.'s, calculated in this case from the means of the oxygen and carbon dioxide values, are of limited significance. Fig. 1 contains examples of some individual records of oxygen uptake and carbon dioxide output in normal and blocked animals.

The measurements of carbon dioxide output in these animals commenced 30 min. after the application of the first coat of emulsion paint to the pleopods. We do not know how the rate varied during this period, but since the initial measured rates were not far short of the rates for normal animals it seems reasonable to conclude that blocking the pleopods had but little immediate effect on carbon dioxide excretion: the subsequent drop would follow naturally as a result of reduced oxygen

uptake. We do not know the permeability of dry emulsion paint to carbon dioxide, but it is unlikely to be as permeable as a moist integument. The simplest explanation of the above results may therefore be that carbon dioxide is normally excreted through the whole integument, including the pleopodal surfaces. This conclusion is consistent with the results of work by Thorpe (1928) and others on insects.

Reinders (1933) found the rate of carbon dioxide output in *Porcellio scaber* to be unaffected by blocking the pseudotracheae (which are on the first two pairs of pleopods only) with paraffin. Oxygen consumption was, however, reduced, and he found an R.Q. of 1·46, as compared with the normal R.Q. of 0·72. He, too, explained the high R.Q. by assuming that carbon dioxide is normally excreted by the whole integument.

There are, in fact, many records of increased R.Q.'s in animals wholly or partly deprived of oxygen (refs. in Prosser, 1952), and these are generally thought to indicate the use of oxygen already present in the tissues or body fluids of the animals concerned, together with the excretion of carbon dioxide already formed. No doubt such processes explain the present results in part, but it is rather difficult to understand why carbon dioxide excretion remains higher than oxygen uptake for so long. Bosworth, O'Brien & Amberson (1936) found a similar sustained rise in the R.Q. of the decapod *Homarus* in low oxygen tensions. These authors used the Van Slyke method of gas analysis, and their animals were, of course, in water. They were able to demonstrate that the high CO_2 figures they obtained were caused by respiratory CO_2 reacting with the calcium carbonate of the integument (from outside) to liberate twice as much bicarbonate. Such an explanation is not applicable to the present results, which must remain unexplained for the time being.

(*b*) *Respiration in dry air*

The use of any manometric method for the measurement of respiration in dry air leads to false results unless the effect of evaporation of water from the respiring animal is allowed for. In the present work the difficulty was overcome by running control experiments with dead animals whose respiratory enzymes had been destroyed by heat. It was established that the rate of evaporation of water from such animals did not differ significantly from normal. An equal number of control and experimental determinations were made for each set of conditions, and the difference between the mean value for the controls and the mean for the experimental determinations was taken as the true respiration rate in the conditions concerned.

In these experiments the procedure before placing the animals in the respirometer cups was as follows: they were weighed, painted if necessary (two coats separated by 15 min. in dry air over phosphorus pentoxide) placed in a slowly moving stream of dry air at 25° C. for 30 min. to make sure that all surface moisture was removed, and tipped straight into the respirometer cup which contained a layer of calcium chloride on the floor and through which a stream of dry air had been passing up to the moment when the animals were introduced. This thorough preliminary drying was found to be important, for the respiration rate of animals placed directly in still dry air without preliminary drying was found not to differ

from that of animals in moist air. This point is referred to again in the discussion on p. 266. During measurement the animals were allowed to come into contact with the calcium chloride. Contact could be prevented by enclosing them in gauze cages, but this was laborious and made no difference to the respiration rate, so that it was discontinued.

When oxygen absorption was to be measured, a saturated solution of potassium hydroxide on filter-paper was used to absorb the carbon dioxide, such a solution comes into equilibrium with a water vapour pressure of about 2·6 mm. Hg (15 % R.H. at 20° C.). (This was of course allowed for by the control experiments.) When carbon dioxide was to be measured, potassium hydroxide was omitted. Measurements were again made for 1 hr. periods, but oxygen and carbon dioxide measurements had to be made on separate groups of animals, since survival was greatly curtailed.

Table 2. *The rates of respiration in mm.^{3}/mm.^{2}/hr.* of woodlice with normal and blocked pleopods in dry air after previous drying in moving dry air at 25° C. for 30 min.*

(Each entry is a mean (± standard error of the mean) of five determinations.)

		Oxygen uptake	Carbon dioxide output	R.Q.
Ligia oceanica	Normal	0·020 ± 0·0031	0·020 ± 0·0031	1·00
	Pleopods blocked	< 0·005 —	0·036 ± 0·0040	Very high
Oniscus asellus	Normal	0·023 ± 0·0038	0·029 ± 0·0037	1·26
	Pleopods blocked	0·005 ± 0·0019	0·032 ± 0·0041	6·40
Porcellio scaber	Normal	0·075 ± 0·0057	0·084 ± 0·0112	1·12
	Pleopods blocked	< 0·005 —	0·045 ± 0·0035	Very high
Armadillidium vulgare	Normal	0·081 ± 0·0064	0·094 ± 0·0143	1·16
	Pleopods blocked	< 0·005 —	0·067 ± 0·0072	Very high

* See footnote to Table 1.

The results of these experiments are shown in Table 2. Five determinations were made for each species in each condition; the figures obtained were very variable (which is not unexpected, for the animals were often approaching exhaustion), but certain main conclusions may be drawn from them.

So far as intact animals are concerned, the rate of oxygen absorption was reduced; to a great extent in *Ligia* and *Oniscus*, and to a lesser extent in *Porcellio* and *Armadillidium*. This confirms the suggestion made by several authors on grounds other than direct measurement, that the presence of pseudotracheae is an advantage so far as respiration in dry air is concerned. The R.Q. of intact animals taken over the whole hour was higher than normal, but since oxygen uptake and carbon dioxide output had to be measured on different animals, and since the variability of each measure was high, little reliance can be placed on these R.Q. values.

All these animals were alive at the end of the experiments (although *Ligia* and *Oniscus* usually appeared sluggish) and if they were removed to moist air, they recovered permanently.

In the blocked animals, oxygen uptake was found to be reduced almost to vanishing point. Carbon dioxide output was also low, but always measurable. R.Q.'s for these animals were often extremely high, but this is simply a reflexion of the very low oxygen uptake. From the fact that oxygen uptake by blocked animals is very much lower in dry air than in moist, it may be concluded that dry air inhibits cutaneous uptake.

Now a few of the blocked animals in dry air, although alive when the measurements started, did not survive the hour in the respirometer cups, and this raises the question as to whether loss of vitality was caused by lack of oxygen, or by some other factor associated with dehydration. This question will be discussed below, after further evidence has been presented.

(*c*) *The effect of high oxygen tension on respiration in dry air*

The oxygen uptake of normal and blocked animals was measured in a thoroughly dry mixture of air and oxygen in approximately equal proportions. A stream of this mixture was caused to flow through the respirometer cups for a few minutes immediately before their attachment to the respirometer. In all other respects the conditions were the same as those for respiration in dry air described in the preceding section.

The results (Table 3) show that with the exception of normal *Armadillidium* there was a greater oxygen uptake in these conditions than there was in dry air, and this is true for both normal and blocked animals. In the former, absorption may have occurred, of course, through the pleopods and/or pseudotracheae; in the latter, absorption can only have occurred through the integument. All these animals, both normal and blocked, were alive at the end of the experiment.

Table 3. *Oxygen uptake in mm.*^{3}*/mm.*^{2}*/hr.* by woodlice in a dry* 50% *air/oxygen mixture after previous exposure to moving dry air at* 25° C. *for* 30 *min.*

(Each entry is a mean (±standard error of the mean) of five determinations.)

	Normal	Pleopods blocked
Ligia oceanica	0·102 ± 0·0053	0·084 ± 0·0072
Oniscus asellus	0·045 ± 0·0030	0·037 ± 0·0033
Porcellio scaber	0·080 ± 0·0052	0·053 ± 0·0046
Armadillidium vulgare	0·081 ± 0·0083	0·040 ± 0·0114

* See footnote to Table 1.

(*d*) *Survival of normal and blocked animals in moist and dry conditions*

The experiments already reported have shown that oxygen may be absorbed through the general integument with greater or less facility by all four species examined. The following experiments were designed to find out for how long such cutaneous respiration is capable of supporting life.

All four species were exposed in the same sets of conditions as those used previously, to determine the period of survival (except that the animals were finally placed in desiccators rather than respirometer cups); the survival of blocked

animals being calculated from the time of application of the first coat of paint to the pleopods. In addition, blocked and normal animals were exposed to moist nitrogen.

At least twenty animals were used to determine the mean survival period in each condition, and the results are shown in Table 4. Variability was again rather great, but the main effects of the various treatments are clear enough. All species survived

Table 4. *Survival times (in hr.) of normal and blocked woodlice when subjected to various conditions*

(Each entry is a mean (±standard error of the mean) of at least twenty records).

	Moist nitrogen	Moist air	Dry air	Dry 50% air oxygen mixture
Normal				
Ligia oceanica	1·6±0·20	*	7·6±0·51	11·0±0·74
Oniscus asellus	1·7±0·21	*	4·5±0·31	9·3±0·51
Porcellio scaber	0·8±0·08	*	10·1±0·83	14·4±1·5
Armadillidium vulgare	0·9±0·07	*	12·3±1·2	18·0±1·3
Pleopods blocked				
Ligia oceanica	1·8±0·09	60±5·3	3·5±0·17	8·0±0·43
Oniscus asellus	1·7±0·12	48±3·7	2·5±0·19	3·3±0·12
Porcellio scaber	0·5±0·02	5·0±0·30	1·9±0·08	6·0±0·40
Armadillidium vulgare	0·5±0·02	4·5±0·25	2·6±0·22	7·1±0·53

* Indefinitely

significantly ($P<0·01$) longer in moist air than in moist nitrogen when the pleopods were blocked, and this is consistent with the conclusion drawn above that oxygen may be absorbed through the integument. But no animals survived indefinitely if the absorption of oxygen through their pleopods was prevented. In other words, cutaneous absorption was insufficient to supply the minimum oxygen requirements.

In dry air, survival was significantly curtailed in all species, but if the oxygen tension was raised, survival was again prolonged. (Oxygen absorption, it will be recalled, was also found by direct measurement to be greater if the oxygen tension was raised.)

When blocked animals belonging to the different species are compared, there appears to be a general correspondence between period of survival and facility for cutaneous uptake of oxygen. Thus in moist air, blocked *Ligia* survived for a mean period of 60 hr., and *Oniscus* for 48 hr. In sharp contrast with these are *Porcellio* and *Armadillidium*, which survived for 5·0 and 4·5 hr. respectively.

In dry air, there is less difference between the survival times of blocked animals of the four species than there is in moist air, and this is consistent with the fact that in dry air cutaneous absorption is almost entirely inhibited in all species.

(*e*) *Does cutaneous absorption of oxygen occur in natural conditions?*

The results considered above leave little doubt that cutaneous respiration is possible in certain circumstances, but there is no means of telling whether, when oxygen is not in short supply, it enters through the general integument as well as

through the pleopods. Such entry has so far been demonstrated only when there is a shortage of oxygen in the tissues and therefore a steep gradient in oxygen tension across the integument.

An attempt was therefore made to demonstrate cutaneous absorption of oxygen in intact animals by injecting reduced indigo, which turns blue in the presence of oxygen. Remy (1925) used this method for several invertebrates, including woodlice. He obtained distinct blueing of the pleopods but he did not record blueing elsewhere. In our experiments, injected animals were fixed in alcohol, embedded in ester wax and sectioned at 15 μ. Counterstaining with carmalum provided a pink ground against which the blueing could be more easily seen. There was intense

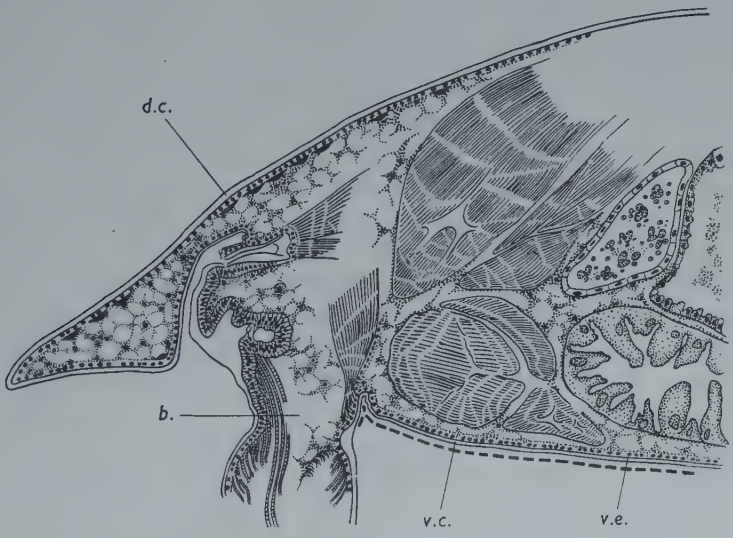

Fig. 2. Transverse section of the left half of the pereion of *Ligia oceanica* previously injected with reduced indigo. The broken line below the ventral integument indicates the region in which the epidermis was blue. *b.*, base of leg; *d.c.*, thick dorsal cuticle; *v.c.*, thin ventral cuticle; *v.e.*, ventral epidermis.

blueing of the exopodites of *Ligia*, the pseudotracheae of *Porcellio* and the endopodites of *Oniscus*. (*Armadillidium* was not used in these experiments.) There was also a less intense, but distinct, blueing of the epidermis of the ventral surface and of the base of the legs in *Ligia*, and of the ventral surface of the abdomen in *Oniscus* and *Porcellio*. Fig. 2 shows, semi-diagrammatically, the area of blueing in *Ligia*. It may be concluded, then, that cutaneous absorption of oxygen does occur in natural conditions. That the amount of blueing observed was not great is hardly surprising, for these animals were intact, and oxygen was being absorbed normally through the pleopods at the time of injection; furthermore, the total area of the integument is large compared with that of the pleopods, and the relatively small amount of oxygen absorbed is likely to be spread out over this area, so that an intense blueing, associated with a high concentration of oxygen in a small area, as in the pleopods, was not to be expected.

DISCUSSION

Cutaneous respiration is by no means uncommon in arthropods. It has been demonstrated in several aquatic forms, and Thorpe & Crisp (1947) found that in the 5th-instar nymph of the bug *Aphelocheirus* oxygen uptake was entirely cuticular. Fraenkel & Herford (1938) found that in the larvae of *Calliphora erythrocephala*, *Chaerocampa elpenor* and *Tenebrio molitor*, oxygen uptake was possible through the general integument when the spiracles were blocked, and amounted to about 25% of the normal basal values. In *Culex* larvae, the amount of oxygen so absorbed was higher still.

The literature on respiration in woodlice contains very few references to the possibility of non-pleopodal absorption of oxygen. Verrier (1932) believed that the epipodial sacs attached to the mouthparts of *Hemilepistus reaumuri* had a respiratory function, but this was not confirmed when Macagno (1938) investigated the matter experimentally by injection of reduced indigo. Macagno (1939) also claimed that in *Tracheoniscus* the pleopods are the only sites of oxygen absorption, on the grounds that no other area turned blue on injection of reduced indigo. We have in the present work observed blueing in regions other than the pleopods, but only when the animal has been exposed to very moist air, and exposure of the animals in Macagno's experiments to dry air is a possible explanation of the difference between his results and ours. Reinders (1933) measured the oxygen uptake of *Porcellio* and found that if the four pseudotracheae were blocked (by paraffin) this was reduced to 39% of normal, thus allowing for the possibility of absorption through the general integument as well as through the other pleopods.

The present work has shown that, in moist air, woodlice are capable of absorbing oxygen through the general integument, and the question raised above (p. 262) concerning respiration in dry air must now be considered. The rates of oxygen uptake and carbon dioxide output are both unaffected if blocked animals are exposed to relatively gentle desiccation in still, dry air, and this may be interpreted as meaning that water lost from the integument is replaced from within rapidly enough to maintain permeability of the integument to oxygen. But after more vigorous desiccation, in a stream of warm dry air, oxygen absorption is greatly reduced, there is apparent loss of vitality, and indeed some of the animals died during exposure. The question is whether the observed loss of vitality was caused by a lack of oxygen or by something else, such as dehydration of the tissues, after which the fall in oxygen absorption followed as a result.

There is evidence that such early loss of vitality would not have occurred if oxygen had been more freely available in the tissues: thus blocked animals, when in dry air with a high oxygen tension, survived longer and absorbed more oxygen. Normal animals showed the same response. Again, blocked animals can hardly have suffered greater desiccation than those of the same species with free pleopods when both were subjected to the same dry conditions, yet the latter absorbed more oxygen than the former and lived longer. The implication of this is that the blocked animals would have used more oxygen if it had been available in the body. It can

therefore be said that these animals in dry air died because they could not obtain sufficient oxygen. On the other hand, it might also be argued that if more water had been available they might have lived longer (or suffered less loss of vitality) even with the same low oxygen uptake. The question as to which of the two factors caused death is not a very useful one: both are involved, and the level of oxygen deficit necessary to cause death will vary according to the degree of dehydration, and vice versa.

Nevertheless, it may properly be said that if they are exposed to sufficiently vigorous desiccation, woodlice are not able to absorb as much oxygen as they need and as they otherwise would. This reduction may well be the result of drying of the integument, making it less permeable to oxygen. But this is not the only possible explanation: there might, for instance, be an active secretion of oxygen across the epidermis which breaks down if the epidermal cells suffer dehydration.

The fact that desiccated woodlice absorb less oxygen than they need, and in this sense at least may be said to die from asphyxia, is relevant to experiments designed to test the relation between survival and humidity. Thus Waloff (1941) did not find a linear relation between period of survival and humidity in *Oniscus*, *Porcellio* or *Armadillidium*, all of which lived for a shorter time in low humidities than would be expected if such a relation were true. This may perhaps be put down to the fact that in low humidities evaporation occurs rapidly, drying the integument and pleopods, so that lack of oxygen may have contributed to the animals' death. Again, Webb-Fowler (unpublished) has found that when woodlice are desiccated, the amount of water which they lose before death is not constant but depends upon the speed of desiccation; and in this case too, lack of oxygen probably contributes to the death of the rapidly desiccated animals.

It has been known for a long time that woodlice are very sensitive to desiccation; more so, in fact, than most insects. The present work has shown one way in which desiccation affects them, namely, by inhibiting the absorption of oxygen. There is evidence in the present work that pleopodal respiration is supplemented by cutaneous respiration to a considerable extent in *Ligia*, and to a lesser extent even in animals with less permeable integuments such as *Porcellio*. In the absence of efficient internal respiratory surfaces (such as those found in insects) a moist integument, general as well as pleopodal, is necessary, and the animals are therefore confined to moist surroundings not only to prevent undue depletion of the water content, but also to ensure efficient respiration.

As regards the evolution of the group, there may have been two factors favouring the retention of a permeable cuticle: first, the ability to reduce body temperature in an ecological crisis (Edney, 1953); and secondly, the need to absorb oxygen. These two factors would be balanced against the obvious advantages of an impermeable cuticle, but it is only after the problems of efficient respiration and tolerance of high temperatures have been solved that the integument can become impermeable, and a true exploitation of the terrestrial environment can begin.

SUMMARY

1. Oxygen uptake and carbon dioxide output were measured in the woodlice *Ligia oceanica*, *Oniscus asellus*, *Porcellio scaber* and *Armadillidium vulgare*, in moist and in dry air, using normal animals and animals whose pleopods had been blocked by emulsion paint.

2. In moist air, the rate of oxygen uptake which occurred through the general integument in animals whose pleopods had been blocked was about 50% of normal in *Ligia* and *Oniscus*, but less than this in *Porcellio* and *Armadillidium*.

3. After exposure to moving dry air, oxygen uptake in dry air was below normal in intact animals and nearly ceased in animals with blocked pleopods. Both showed a somewhat higher uptake if the oxygen tension was raised.

4. Carbon dioxide output in moist air was not immediately inhibited by blocking the pleopods, suggesting free diffusion through the integument. Output fell during the first hour, but the R.Q. remained above 1 in all species.

5. Survival times of blocked animals in moist air corresponded qualitatively with rates of oxygen absorption in the four species. In dry air survival was further curtailed, but prolonged again by an increased oxygen tension. Blocked animals of all species survived for a shorter time in moist nitrogen than in moist air.

6. Injection of reduced indigo showed that oxygen was absorbed by intact animals through the thin ventral integument and bases of the legs in *Ligia*, and to a lesser extent, through the ventral abdominal integument in *Oniscus* and *Porcellio*.

7. The ecological implications of these results are discussed, particularly with reference to the relation between survival and humidity. The dehydration death point is probably affected by the level of oxygen deficit and vice versa. A water-permeable integument is of value for respiration as well as for temperature control, but restricts the habitat range of terrestrial animals.

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THE FUNCTION OF THE LYRIFORM ORGANS OF ARACHNIDS

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INTRODUCTION

The term 'lyriform organs' was first used by Gaubert (1890) to describe the groups of peculiar sense organs found on the legs and elsewhere in arachnids. The individual sensilla in these organs are similar in structure to isolated sensilla which occur widely on the cephalothorax and abdomen of many spiders and other arachnids and which were first described by Bertkau (1878). They are often referred to as 'slit sense organs' (Spaltsinnesorgane), since their appearance in surface view suggests a slit in the cuticle. Recent investigators (Vogel, 1923; Kaston, 1935) are, however, agreed that there is no actual canal leading from within to the exterior, but merely a pronounced thinning of the 'chitin' along a narrow groove with a continuous epicuticular membrane. The term 'lyriform organ' describes the characteristic appearance of the compound organs where a number of slits of varying length are arranged in a parallel or near-parallel orientation in the same manner as the strings of the musical instrument (Fig. 1).

Single or compound sense organs of this type occur in nearly all arachnids, and their distribution over the body is remarkably constant within each order (Gaubert, 1892; Hansen, 1893; McIndoo, 1911). They are found near the joints of the legs and other appendages, on the sterna of the cephalothorax and abdomen and on the sting of scorpions, and, as Vogel (1923) has pointed out, are constant not only in position but also in the orientation of the slits. The leg organs are usually compound and are often situated where the cuticle is curved near the articular surfaces.

The internal histological structure of the sensilla has been described fully by Vogel (1923) and Kaston (1935). In external view the slits (10–50μ long) have a slight widening at one point and to the centre of this widened region inserts the distal process of a sense cell. The cells of the hypodermal layer under the sensilla are modified in appearance and are probably responsible for the modified cuticular structure of the region.

The most recent view of the function of the slit sense organs (Millot, 1949) is that they are chemoreceptors, an opinion supported by McIndoo (1911) and Kaston (1935). Apart from earlier implausible hypotheses the only other function attributed to them is that of proprioception (Vogel, 1923). McIndoo and Kaston supported

* The experimental work reported here was carried out during 1953 in the Department of Physiology, University of Ceylon, Colombo, and in the laboratory of the Division of Entomology, Department of Agriculture, Peradiniya, Ceylon.

their hypothesis by experiments on the contact chemical sense of spiders, and claimed that the distribution of the slit sense organs on the body was consistent with their being the structures concerned; Vogel had no experimental evidence to connect them with a kinaesthetic sense, but was impressed by the constancy of orientation and the continuity of the epicuticular membrane, features which he could not correlate with a chemoreceptive function.

None of the workers who have studied these organs appear to have noted the similarity between their structure and distribution (at any rate on the appendages) in arachnids and the structure and distribution of the campaniform sensilla of insects. In both cases modified regions of the cuticle near the joints have sense-cell processes attached to the centre of a thin membrane, and both types of sensillum have a constant orientation in different individuals and in related species. The

Fig. 1. Lyriform organs on the chelicera of a spider, to show the different orientation of the slit sensilla of the two groups (from Vogel, 1923).

campaniform sensilla of insects were at one time thought to be chemoreceptors (McIndoo, 1914), but Pringle (1938), by recording impulses in their sensory nerves, established that they are mechanoreceptors sensitive to strains in the cuticle, and that the orientation of the more advanced type of sensillum found in adult insects is significant in relation to the quality of stimulus to which they respond. Similar investigation of the range of sensitivity of the lyriform organs is made difficult by the small size of the arachnids available in temperate countries and by the poor survival of spiders as experimental animals for neurophysiological work. The tropics are better provided in this respect, and it has now been possible to settle the function of the lyriform organs in an unequivocal manner.

MATERIAL AND METHODS

Two large arachnids have been used in this investigation; a scorpion *Heterometrus* (*Palamnaeus*) *swammerdami* Simon, and an amblypygid *Phrynichus lunatus* (Pallas) (Fig. 2). The animals were collected in the field in Ceylon and kept in cages in the laboratory. The scorpions varied in body length from 7 to 15 cm. Three specimens only of *P. lunatus* were obtained; these had a body length of about 3 cm.

Impulses were recorded in various sensory nerves by means of platinum wire electrodes, a Grass Type P.4 pre-amplifier and a Cossor Type 1049 oscilloscope. Alternatively, the output from the pre-amplifier was fed to an M.S.S. Type PMR/1 magnetic tape recorder and the results re-photographed on oscilloscope film after return to England. This method distorts the waveform of nerve impulses but is satisfactory when only the frequency and relative amplitude of impulses is of interest. Since the motor speed of the tape recorder varies slightly with the voltage of the electric supply, time measurements from oscilloscope records made in this way have an accuracy of about ± 10%.

No form of Ringer's solution was necessary. The temperature of the laboratory was 28–32° C.

Fig. 2. *Phrynichus lunatus* (Pallas). (Two-thirds natural size.)

RESULTS

If electrodes are placed on the nerve trunk near the base of the amputated leg of a scorpion impulses are readily recorded in the sensory fibres. The long setae which occur on each of the leg segments give rapidly adapting impulse discharges of large amplitude when they are moved, and a confused pattern of small impulses results from touch stimuli to the distal tarsal segments.

Forced movement at the coxo-trochanteral, trochantero-femoral, femoro-tibial or tibio-tarsal joints gives a different result, the discharge then consisting of impulses in a small number (1–6) of fibres, with slow adaptation (Fig. 3A–C). The fibres concerned appear to be of various sizes and different fibres are active when the movement is one of extension from those reacting to flexion. Slow forced movements bring in only the smaller fibres, while fast movements usually excite also a single large fibre, also different for the two directions of movement. At the coxo-trochanteral and trochantero-femoral joints the fibres reacting to joint movement run centrad up the leg for a short distance as a separate trunk before joining the main nerve, and clearer records are obtained when the electrodes are placed on these small trunks.

Similar results are obtained from the femoro-tibial joint of the claw (pedipalp) and legs of the amblypygid *Phrynichus lunatus* (Fig. 4). Here again slow forced movement of the joint excites only one or two small fibres and fast movements bring in also a larger fibre, both discharges occurring in different fibres for the two directions of movement.

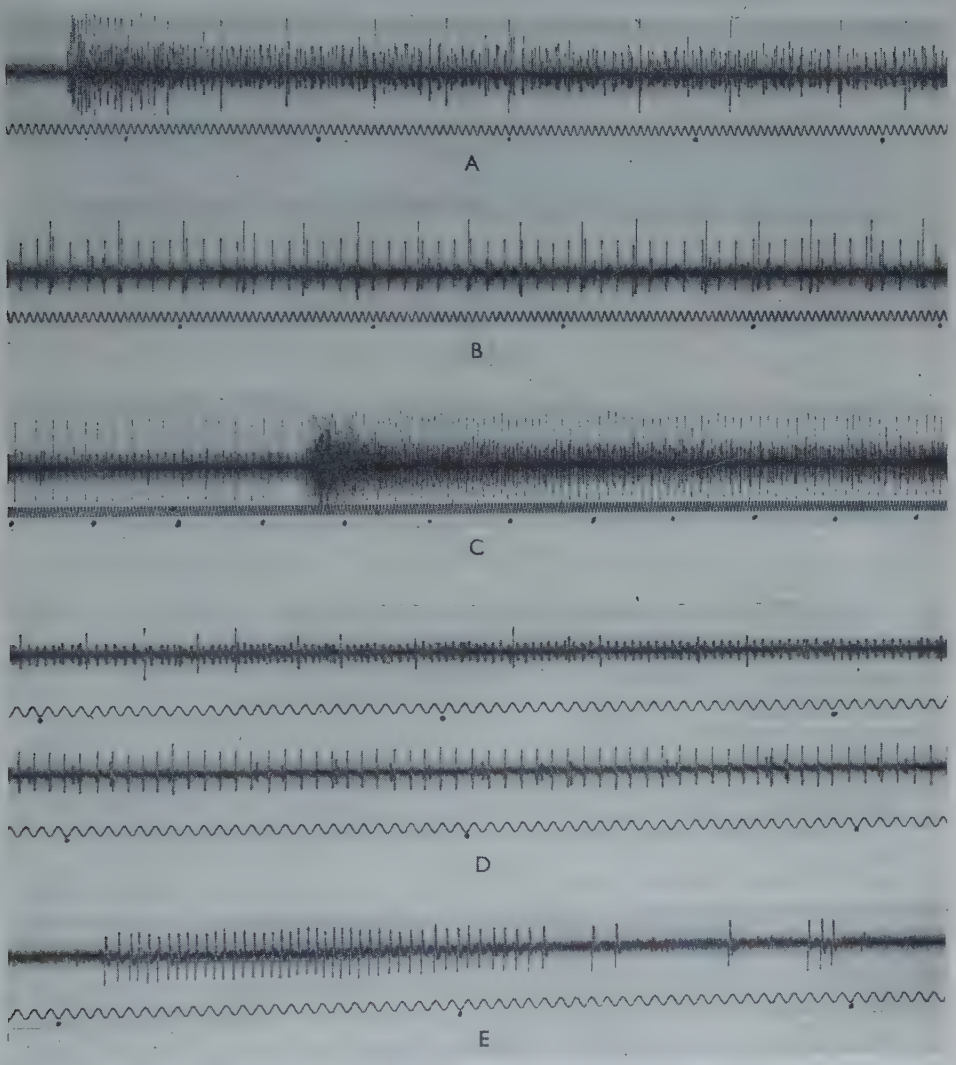

Fig. 3. Oscilloscope records from scorpion sensory nerves, re-printed from magnetic tape recordings. A: leg nerve at base of femur; forced extension of femoro-tibial joint; B: small nerve trunk in trochanter, maintained forced flexion of trochantero-femoral joint; C: small nerve trunk in coxa, increase from partial to complete extension of coxo-trochanteral joint; D: leg nerve at base of femur, pressure with needle at two different places on distal end of femur, portions of a continuous record under conditions comparable to A; E: abdominal nerve, pressure with needle on terminal segment of sting. Time marker 50 cyc./sec. (trace) and 0·5 sec. (dots).

In one experiment on the coxo-trochanteral joint of the scorpion leg the motor nerve supply to the flexor trochanteris muscle was left intact and periodic flexor movements occurred spontaneously. Small diameter nerve fibres from endings at the joint were excited by these movements but the discharge was considerably less than that produced by forced movement of the same amplitude. No impulses in large sensory fibres occurred during spontaneous flexion.

Attempts were made to locate the endings responsible for the sensory discharge accompanying joint movement. With the parallel in mind of the campaniform

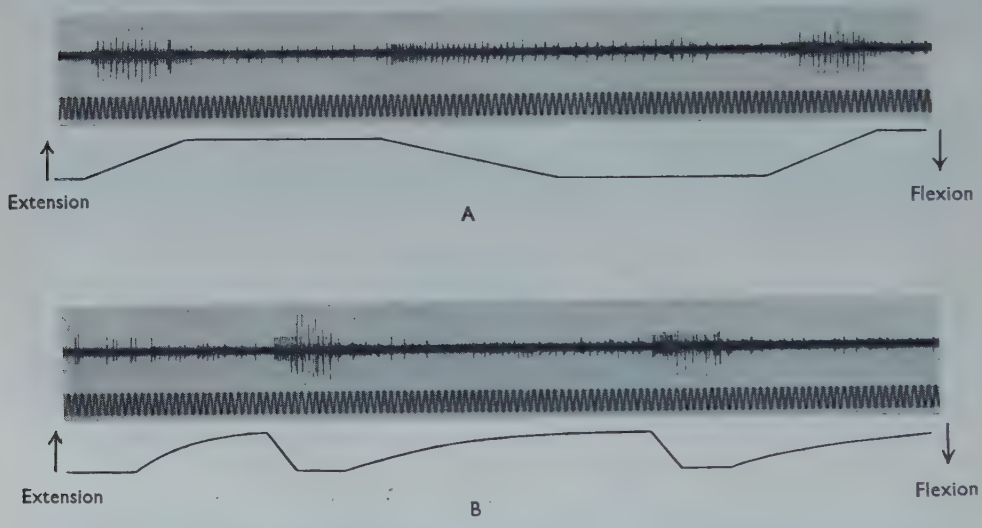

Fig. 4. Oscilloscope records from *Phrynichus* claw nerve; forced movement of femoro-tibial joint at various speeds. A, fast extension with slow flexion; B, very fast flexion with slow extension. Time 50 cyc./sec.

sensilla of insects, which are strongly excited by strains induced by any form of distortion of the cuticle near the joints (Pringle, 1938), pressure was applied with the blunt point of a needle to the end of the femur near the femoro-tibial joint of the scorpion leg. Fig. 3D shows that a considerable discharge from slowly adapting endings can be obtained in this way, the size of the fibres so excited being comparable to that of the smaller fibres which are excited by forced joint movement. It is noteworthy that discharges in different fibres were obtained by pressure with the needle at slightly different locations on the femur. This sensitivity to distortion of the cuticle of the next proximal segment was confirmed for each of the four basal joints of the scorpion leg and in the claw and legs of *Phrynichus*. A similar result was also obtained from the abdominal nerves of the scorpion in response to pressure anywhere on the terminal segment of the sting (Fig. 3E).

These results point clearly to the lyriform organs as the structures concerned in the discharge of the smaller impulses during forced joint movements and show (as postulated by Vogel, 1923) that the slit sense organs are mechanoreceptors, sensitive to strains in the cuticle. Groups of sensilla, sometimes lyriform in appearance,

are present on the scorpion leg near the joints close to the positions of maximum sensitivity to pressure with a needle (Fig. 5). The experiment on spontaneous flexion suggests that, as with the campaniform sensilla of insects, the endings are more strongly excited by forced movements of the joints (or presumably by resisted active movement) than by spontaneous movement when the leg is out of contact with the ground. Vogel's suggestion that the slit sense organs confer a kinaesthetic sense analogous to the muscle and tendon senses of vertebrates is therefore confirmed.

The possible chemoreceptive sensitivity of the slit sensilla was tested briefly in scorpions by the application of liquid xylol to the region of the femoro-tibial joint; no sensory discharge resulted from this treatment.

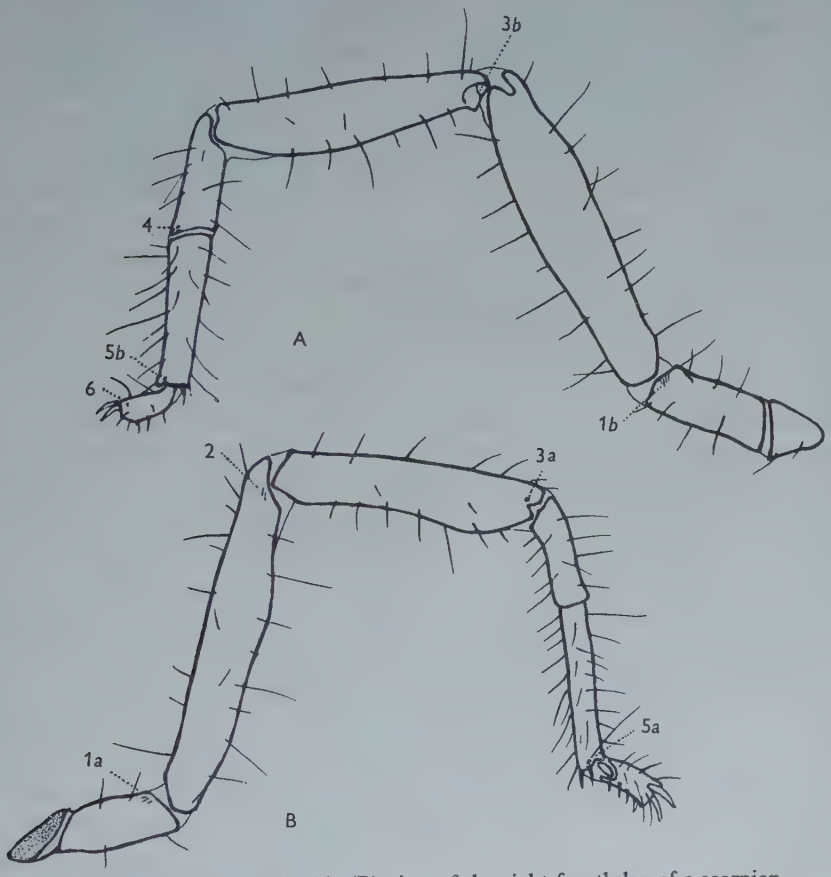

Fig. 5. Outside (A) and inside (B) view of the right fourth leg of a scorpion, showing the position of the slit sensilla (groups numbered 1–6).

DISCUSSION

Two unresolved points may be mentioned. The sensory discharge in large fibres which occurred, both at the three basal joints of the scorpion leg and at the femoro-tibial joint of *Phrynichus* claw, on rapid forced movement of the joint shows several

features which appear to distinguish it from the discharge in smaller fibres which has been identified with the slit sensilla of the lyriform organs. These large impulses adapt more rapidly than those in smaller fibres and occur only with rapid forced movement. Fig. 4 also shows that the discharge of large impulses regularly starts after the maximum frequency in the smaller fibres when some movement of the joint has taken place. If the ending responsible for the large fibre discharge is also a slit sensillum it would be expected that it would be excited at the moment of maximum strain in the cuticle when the frequency of small impulses is also maximal. It is also noteworthy that the large impulses could never be obtained by pressure with a needle on the cuticle near the joint, although impulse frequencies in small fibres were often higher with this form of stimulation than with very rapid movement. On the other hand, the large fibre discharge could usually be obtained without simultaneous excitation of the smaller fibres by probing with the needle into the soft flexible regions of the intersegmental membranes. It was also found that the large fibre discharge on rapid movement of the femoro-tibial joint (scorpion) was selectively abolished by section of the tibia half-way along its length, while the small fibre discharge remained unaltered. The conclusion from these experiments was that this large fibre discharge originates from an ending, of a different type from the slit sensillum, situated internally in the leg near the joint; a structure which may be responsible was discovered by dissection and will be described in a subsequent paper (Parry & Pringle, in preparation).

The other point concerns the direction of strain to which the lyriform organs respond. Pringle (1938) concluded that the campaniform sensilla of insects were excited by a compression strain parallel to the long axis of the sensillum (which is elliptical but rarely has a major/minor axis ratio of more than 3:1). This conclusion was reached partly on microanatomical grounds, the structure being such that this stimulus would be expected to stretch the nerve ending (Fig. 6), and partly from the positioning and orientation of the sensilla on the legs, a compression strain parallel to the long axis being present when the leg is in contact with the ground. Similar reasoning is difficult to apply to the slit sensilla of arachnids owing to the fact that they are usually situated in strongly curved regions of the cuticle. It is, however, highly probable that a greatly elongated structure of this sort is selectively sensitive to one direction of strain, and the differences in orientation of neighbouring lyriform groups, when two organs occur in close proximity (Fig. 1), suggests that such a qualitative discrimination is a property of the sensilla. The fact that different endings are excited when pressure is applied with the needle at slightly different places on the cuticle has already been noted. If it is assumed, as for the campaniform sensilla, that stretch of the distal process of the sense cell is the adequate stimulus for excitation, then Fig. 6 shows that the sensilla should respond when the tension component of shear is at right angles to the orientation of the slits. In a plane surface of uniform thickness this implies a compression component parallel to the slits and a similarity in sensibility to the insect campaniform sensillum; but it is probably unwise to apply reasoning derived from the properties of uniform plane surfaces to the irregular and highly dissected surface of the arachnid cuticle

at the lyriform organ. No certain conclusion can be reached on this point without accurate model-building, an undertaking which would be rendered extremely difficult by the differences not only in thickness but also in elastic rigidity of different parts of the cuticle near the articular surfaces of the joints.

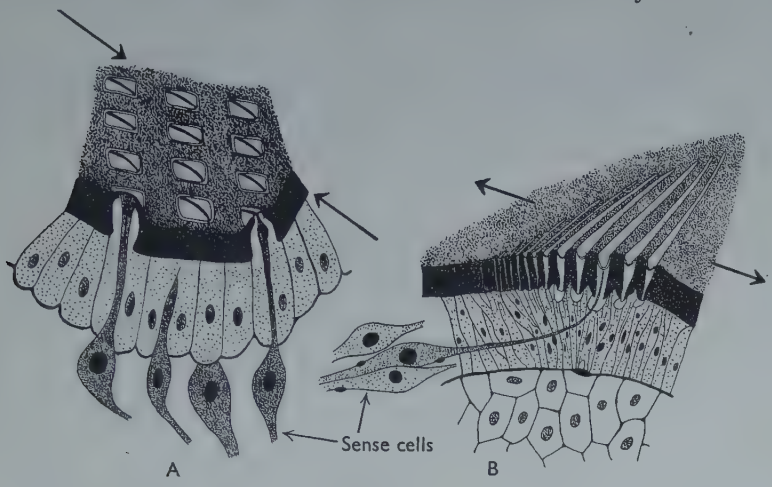

Fig. 6. Diagrammatic drawings of the structure of an insect campaniform sensillum (A) and an arachnid slit sensillum (B). The arrows show the probable direction of strain which excites the sensilla. (A: based on drawings of the basal plate sensilla on the haltere of *Calliphora* (Pflugstaedt, 1912); B: based on a drawing of the lyriform organ on the patella of a spider (Vogel, 1923).)

SUMMARY

Experiments involving the recording of impulses in sensory nerves in the appendages of a scorpion and an amblypygid show that the slit sensilla (lyriform organs) of these arachnids are mechanoreceptors, sensitive to strains in the cuticle and analogous to the campaniform sensilla of insects.

I am grateful to Prof. Koch, Department of Physiology, Colombo, and to the Director of Agriculture, Peradiniya, for giving me facilities to carry out this work in Ceylon; and to the Director of the National Museum, Colombo, for allowing his assistant to collect material for my experiments. The visit was made possible by the award of a Leverhulme Research Fellowship, and I also received financial assistance from the H. E. Durham Fund of King's College, Cambridge, and from the British Council. Part of the apparatus used was purchased with the aid of the Grants Committee of the Royal Society.

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FACTORS AFFECTING THE TEMPERATURE EXCESS OF INSECTS IN SUNSHINE

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(1) INTRODUCTION

The temperature of an insect is of paramount importance in determining its activity. When heated by the sun its temperature is not the same as that of the air but is greater by an amount which we will call the temperature excess. As activity is so dependent on temperature it is of considerable interest to know more about the way in which the various factors operate to control this temperature excess.

The direct field observations so far made on the temperature excess of an insect in the sunshine apply to large forms such as the locust (Buxton, 1924; Strelnikov, 1936; Kennedy, 1939; Gunn, Perry, Seymour, Telford, Wright & Yeo, 1948; Parry, 1951). Calculations such as those by Parry (1951), although they yield quite a reasonable answer for an insect the size of a locust at a considerable wind speed, involve a number of assumptions. There is reason to believe that certain of these would not hold good for most insects of temperate lands which are much smaller, and which are found when resting in positions with very low wind velocities. These wind velocities are certainly below 1 m./sec. and often below 0·1 m./sec. (see Stocker, 1928; Geiger, 1950).

This paper describes laboratory experiments on the temperature excess of insects and plasticine spheres carried out under known conditions similar to those found in microclimatic conditions in the field, with a view to finding the temperature excess to be expected under natural conditions, and to elucidating the way in which the factors work at small size and low wind speed.

Now it can be shown that for a small physical body similar to an insect the temperature excess varies directly with the radiation strength; with the absorptivity (which depends on the spectral composition of the radiation and on the colour and characteristics of the body); with a fractional power of the size, and inversely with a fractional power of the wind velocity. Metabolism and evaporation are practically negligible in the resting insect. Uncertainty arises concerning the relations between temperature, wind and size, for at small size and low wind speeds bodies tend to develop their own convection currents and to be controlled by the laws of natural convection rather than forced convection. It is to be expected that small insects in their natural habitat lie in the region of transition between these two types of flow. Of the factors involved in heat loss by convection, variation in the viscosity, density and conductivity of the air may be neglected as their effects over

the normal range of climatic variation are slight. Long-wave radiation between the insect and the background is also small and may be neglected. The main questions which we must consider for a representative range of insects are (i) whether temperature excess varies directly as radiation strength, or whether there are other complicating factors; (ii) what is the range of absorptivity; (iii) to what power of the size the temperature excess may be related, the 0·2 or 0·4 powers being characteristic of natural or forced convection respectively; and over what size range the powers hold good (see Appendix); (iv) to what extent temperature excess is dependent on wind velocity (natural or forced convection); and (v) whether the known heating effects of metabolic activity cause a temperature rise in a way which is strictly additive to that produced by radiative heat gain.

(2) METHODS

(i) *The wind tunnel*

A return-flow wind tunnel (Fig. 1), similar to that used by Ramsay (1935), was constructed, and the insect under observation was mounted facing the flow of wind in the centre of the air stream. Wind speeds in the 10–100 cm./sec. range were obtained by the use of a shunt channel and appropriate shutters to by-pass air from the insect channel. The armature and field of the fan were connected up in the manner used by Hollick (1940) to maintain a steady flow at low velocities. The experiments considered here were all carried out with the air temperature close to, or a few degrees above, that of the laboratory.

Wind velocity was measured by a hot wire anemometer, the element being a 1 cm. length of s.w.g. 50 platinum wire. Off-balance deflexion of the bridge galvanometer

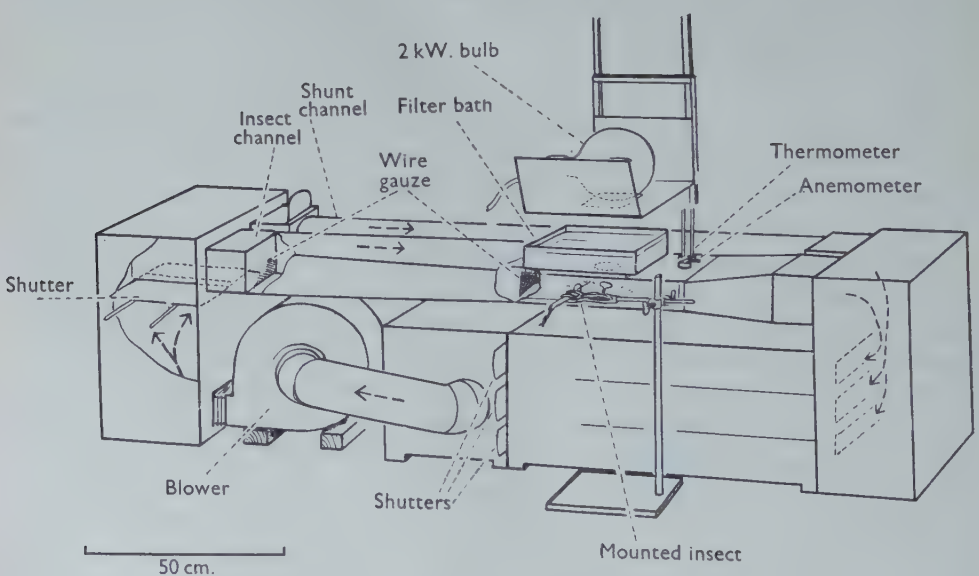

Fig. 1. Return-flow wind tunnel for determination of temperature of mounted insects under known conditions. Broad arrows indicate direction of air flow.

was calibrated outside the tunnel against a smoke puff in a celluloid tube 1 or 2 m. long.

Radiation was provided by a 2 kW. Siemens projector bulb supported above the insect chamber by an arrangement so that it could be raised or lowered at will. The bulb, although rated at 240 V., was under-run at 210 V. to lengthen its life. Metal screens were used to cut off the long-wave radiation from the glass globe not immediately in line with the filament. A trough containing water or copper chloride solution was placed beneath the bulb, between it and the insect chamber, if desired.

(ii) *Radiation measurements*

A compensation instrument (Fig. 2) of the type of the Ångström Pyrheliometer (Ångström, 1899), was constructed for these measurements. The surfaces which received the radiation were two identical strips of manganin, each about 3 cm. long, and blackened with soot. Insulated thermocouples attached to the centre of each strip by a flake of mica were connected up to a galvanometer in such a way that any difference in temperature of the two strips produced a deflexion. In order to measure a given radiation, a shutter was moved so that one strip was exposed to the unknown radiation, while the other was heated by an electric current. The current

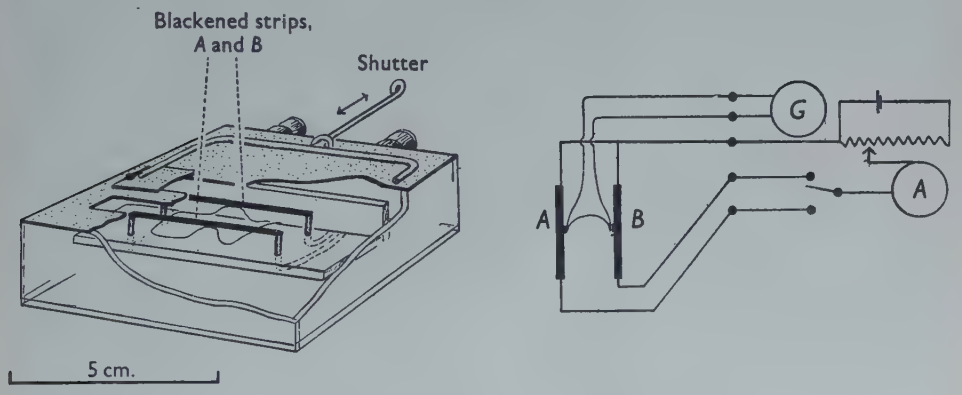

Fig. 2. Details and wiring of pyrheliometer.

was adjusted until the two strips were of equal temperature, when the radiation strength was derived from the ammeter reading and an appropriate constant, itself derived from the area of the strips and their resistance, assuming the blackened surface to absorb 95 % of the radiation irrespective of wave-length.

This particular instrument was constructed to have a large acceptance angle so that it would give an accurate reading when the bulb was close to the strips, and it was of such a shape that it could be placed inside the insect chamber with the blackened strips occupying the position in which the insect was later placed. It was checked against a Michelson actinometer obtained on loan from Kew Observatory, and which Dr G. Robinson had kindly calibrated against one of the Kew Ångström instruments.

Radiation intensity was usually adjusted by varying the height of the bulb, because it is not easy to use filters for reducing radiation which includes infra-red as well as visible without altering the spectral distribution at the same time.

Radiation conditions near the ground are complicated; they have recently been reviewed by Geiger (1950) and Sutton (1953). The most important component, the direct solar radiation, is received from the sun itself and from the small area of sky immediately round about. When the sun is high and the sky clear this may reach a value of up to 1·5 cal./cm.^{2}/min. normal to the surface of measurement (Kimball & Hand, 1936; and see recent Kew values in Stagg, 1950). It is the equivalent of this component which was used in these experiments. Reflexion and long-wave radiation were minimized by surrounding the insect by a black painted insect chamber maintained close to air temperature.

As absorptivity varies with spectral composition, it was necessary to insure that the spectral composition of the radiation used approximated to sunshine. The composition of the direct solar radiation for Washington and Davos, together with that of the radiation used in the experiments is shown in Table 1. The Washington figures are derived from a diagram published by Brackett (1936), itself derived from data by Abbot (1929), the curve taken being that corresponding to air mass 2. The Davos figures are those published by Sutton (1953) for the month of June. Measurements of spectral composition of the light from the bulb after it had passed through the water or $CuCl_2$ solution were made with the pyrheliometer in conjunction with the Chance filters On 20, OY 2 and OR 1, cut-off values being taken from published transmission curves. A depth of 2 cm. N/10 $CuCl_2$ gave a spectral composition close to that of Davos sunshine and a 2 cm. N/20 $CuCl_2$ a composition close to that of Washington sunshine. These depths of $CuCl_2$ had the disadvantage that they intercepted so much heat that a strength of 1·5 cal./cm.^{2}/min. could not be attained in the insect chamber. For this reason, and as subsequent work showed that the spectral composition was of minor importance, a 3·5 cm. depth of water was used. Strictly, this gave a radiation more akin to Washington sunshine with a low sun, air mass 10 (where of course it is associated with a low total intensity).

Table 1. *Percentage spectral composition of sun's radiation at Davos and at Washington, and of the bulb with the various filter baths used in experiments*

	Blue	Yellow	Red	Infra-red
Sunshine at Davos, June	Under 0·56 μ 29·1	0·56–0·63 μ 11·9	0·63–0·76 μ 17·7	Over 0·76 μ 41·2
Sunshine at Washington (average air-mass 2)	Under 0·55 μ 12·9	0·55–0·64 μ 26·8	0·64–0·8 μ 15·4	Above 0·80 μ 44·5
Bulb plus 3·5 cm. water	11·1	12·2	25·8	51·0
Bulb plus 2 cm. N/20 CuCl	15·2	17·1	19·0	48·7*
Bulb plus 2 cm. N/10 CuCl	22·9	24·4	15·3	37·6†

* Nearest Washington. † Nearest Davos.

(iii) *Measurement of the temperature of an insect by a thermocouple*

The thermocouple units used for measuring the temperature excess consisted of two soldered eureka-platinum junctions of s.w.g. 50 wire connected to copper leads passing down a tube which supported the wire to which the insect was attached by soft wax (Fig. 3). In use, one of the junctions was inserted into the thorax of a heavily etherized or recently killed insect so as to lie in approximately the centre, between the muscle bands, and was sealed in position with a small drop of wax, while the other projected forwards into the air stream. These two junctions therefore measured the difference between the temperatures of the thorax and of the air stream. Temperatures are by no means uniform throughout an insect; in Table 2 is

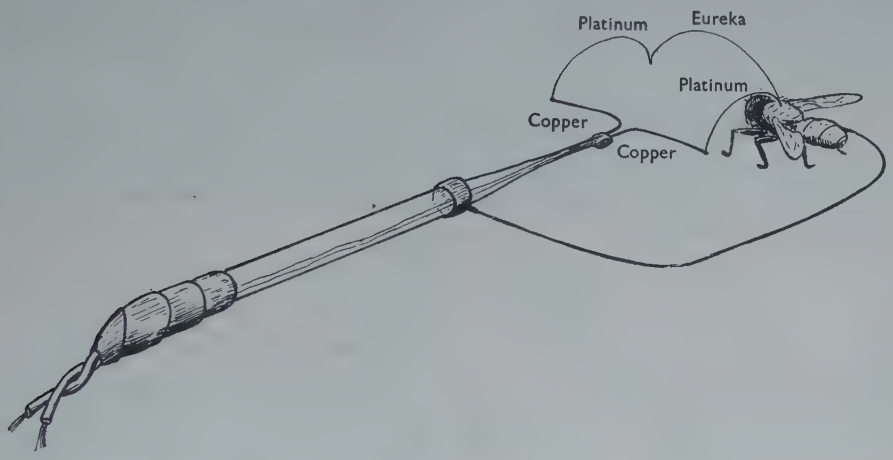

Fig. 3. Details of method of mounting insect with fine wire thermocouple inserted in thorax in such a way as to lie between muscle bands.

Table 2. *Temperature excess* (° C.) *in different parts of insect subjected to constant radiation of* 1·5 *cal./cm.²/min.*

Position of thermocouple	Temperature excess (° C.)				
	Muscina	*Carausius* (Orthop.)		*Schistocerca* (Orthop.)	
		Normal	Blackened	Normal	Blackened
Thorax:					
On upper surface	—	7·3	14·2	10·1	16·5
Very close to upper surface	4·6	—	—	—	—
Above centre of thorax	4·5	7·6	12·0	10·1	13·4
Centre	4·3	—	—	—	—
Below centre of thorax	4·1	(2·52)*	(7·65)*	—	—
Very close to lower surface	4·0	—	—	—	—
On lower surface	—	(4·75)*	(11·2)*	—	—
Abdomen:					
Above centre	6·9	—	—	—	—
Centre	6·15	—	—	—	—
Below centre	6·2	—	—	—	—

* Values arrived at by turning insect upside down.

given the temperature excess found in different parts of three insects when mounted in the air stream at a wind speed of 50 cm./sec. and radiation strength of 1·5 cal./cm.²/min. The considerable differences in temperature between the upper and lower parts of the thorax of *Carausius* probably result from the gut in the centre containing air. The current produced was measured on a Cambridge Instrument Co. pot galvanometer of 40 Ω resistance giving close to 1 cm. deflexion on a scale 1 m. distant for 1° C. difference of temperature between the insect and the air. The measurement of the temperature of an insect by thermocouple wires has given some difficulty in the past; Krogh (1948) showed the heat loss along thermocouple wires can be quite large. With such fine wires, however, heat transfer along the wires was found to be negligible, for neither touching the fine thermocouple wires just outside the thorax of a 3 mm. diameter insect with a hot wire, nor shielding them from the wind by means of a thin cylinder of cellophane or paper produced a difference in galvanometer reading corresponding to more than 0·05° C.

(iv) *Estimation of temperature rise from rate of wing beat*

The temperature rise in small insects such as *Drosophila* cannot readily be determined by the use of a thermocouple of this size when the insects are alive because of their small size.

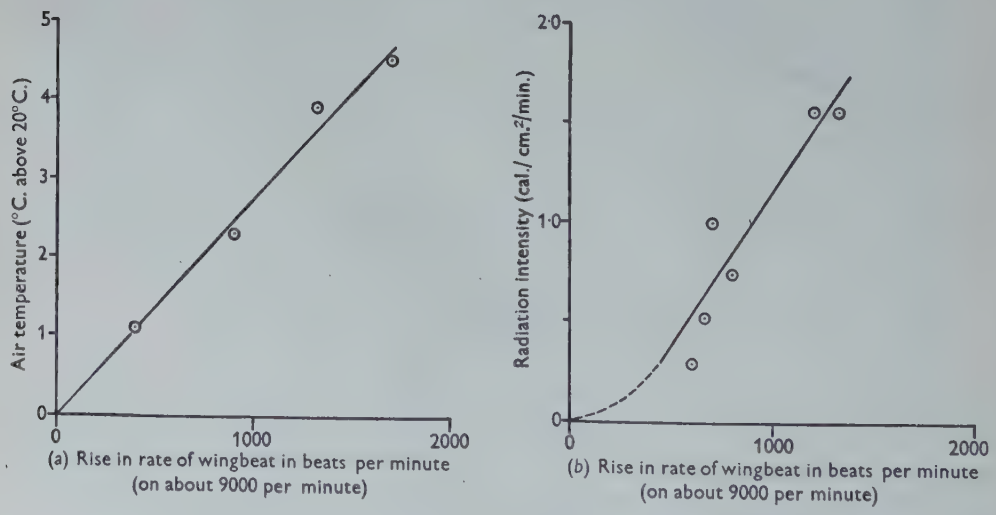

Fig. 4. Variation in increase of rate of wingbeat of *Drosophila* with change in (*a*) air temperature, (*b*) radiation intensity.

It is possible to estimate the temperature rise of flying *Drosophila* when exposed to radiation by observing changes in its rate of wing beat. This has been shown to be proportional to the temperature over the normal range (Chadwick, 1939) and to decrease slowly owing to fatigue or to utilization of reserves (Williams, Barness & Sawyer, 1943). In the present case, the rate of wing beat of a mounted and flying specimen was calibrated against air temperature by transferring it from a tube in

a water-bath at 18° C. to tubes in water-baths at higher temperatures and back again, while the rate of wing beat was followed by an electric stroboscope. Two-minute exposures to each temperature were found to be suitable, observations of wing beat being made every 10 sec. Reference to the rate at 18° C. every 2 min. was necessary to allow correction for alteration in this basal rate due to fatigue. Immediately after calibration the same insect was transferred to the insect chamber in such a way that it was subjected to radiation from above while the rate of wing beat was followed by silhouetting it against the light of the stroboscope. This enabled the relation between wing beat and radiation to be determined. Experiments showed that light unaccompanied by appreciable heat radiation had little or no effect in raising the rate of wing beat apart from a temporary increase in the rate over 10 or 20 sec. on bringing insects from complete darkness to the light. The persistent increase of wing rate in strong radiation is therefore considered to be due solely to temperature. Comparison of the two curves, wing rate against air temperature and against radiation (Fig. 4) indicated that at a wind speed of 50 cm./sec. and radiation of 1·0 cal./cm.^{2}/min. the temperature rise was 2·5° C. in a large wild specimen of *Drosophila* and 1·0–1·5° C. in a small *D. melanogaster*, while in the latter, at a wind speed of 10 cm./sec. (about the normal speed of flight) the temperature rise was 2·5° C.

(3) FACTORS AFFECTING TEMPERATURE EXCESS

(i) *Intensity of radiation*

The variation in temperature excess with radiation strength for six insects is shown in Fig. 5. In the five larger forms the conditions were: wind 50 cm./sec., temperature about 20° C., radiation 0–2·0 cal./cm.^{2}/min. Temperature excess was measured by thermocouple. In *Drosophila*, run at a lower wind speed, temperature excess was assessed by change in rate of wing beat.

The relation was substantially linear in the five larger species. This linear relation is to be expected where the laws of forced convection hold good. Departures from linearity may be expected under conditions of natural convection (where temperature varies as the 0·8 power of the radiation) and where evaporation is important. The temperatures are slightly lower than would be expected at the highest radiation intensities; at these temperatures evaporation is not likely to be involved for the cuticular waxes would retain their protective function (Wigglesworth, 1945), so the discrepancy is probably due to the slight development of natural convection with the increase of buoyancy forces at higher temperatures (see § 3, (v)). In *Drosophila*, the relationship was not linear, but in this case radiation intensity was altered by filters, so non-linearity may have been caused by variation in absorptivity with spectral composition.

(ii) *Spectral distribution of radiation*

The spectral composition of the radiation was varied by inserting copper chloride and water filters between the bulb and the insect. The proportion of infra-red was varied from 8% (less than in daylight) through 40% (daylight) to 78% (greater than daylight), the total vertical radiation strength being adjusted to 0·5 cal./cm.^{2}/min.

each time. In one case, the same radiation intensity was also provided by a domestic electric radiator giving almost entirely infra-red radiation with a peak wavelength at about 2·5 μ.

Five species of flies, *Lucilia caesar* and *Phormia caerulia* with the thorax metallic green and blue, and *Eristalis tenax*, *E. arbustorum* and *Sphaerophoria scripta* with

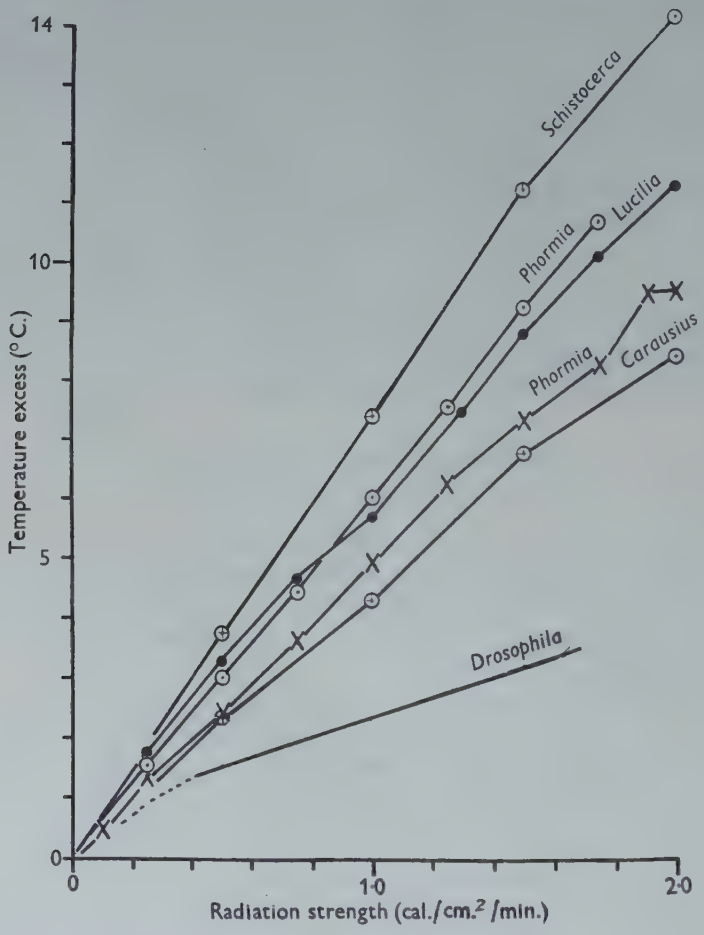

Fig. 5. Variation in temperature excess with total radiation intensity.

the thorax dull brown showed very little difference in temperature (under 5 %) over the above range. Clearly, therefore, any differences in spectral composition in the sun's radiation with the time of the day are of very minor importance to insects of this size.

It is probable that smaller insects may be more sensitive to spectral composition, for they no doubt transmit a larger part of the incident radiation. The absorption bands of flesh of various invertebrates was shown by Rucker (1933*b*) to be closely similar to those of water, and the same no doubt holds true for these forms.

Some indication that this may be so is afforded by *Drosophila*, for in most of the experiments dealing with the relation of temperature (estimated by wing beat) to radiation, the latter was changed by inserting various filters so that at the highest total radiation intensity the light contained the greatest proportion of infra-red (as in the case shown in Figs. 4 and 5). In these cases the more intense radiation (richer in infra-red) produced a smaller increase in wing rate per unit radiation than did the less intense radiation. As the wing beat was more strictly proportional to radiation when the latter was altered by altering the height of the bulb, it probably indicates that a greater proportion of the infra-red than of the visible was passing through the insect.

(iii) *Colour and absorptivity of insect*

The colour of an object affects the proportion of radiation it absorbs, or absorptivity. Temperature excess is commonly proportional to absorptivity.

It is difficult to assess accurately the effect of the colour of an insect on its temperature by finding the temperature excess of similar insects varying in colour alone because no two insects are rarely quite alike apart from colour, and furthermore, it is not even easy to obtain temperatures which agree exactly when the same thermocouple is removed and replaced in the same insect.

Some information on the effect of colour may be gained from the experiments run for the effect of size on temperature (Fig. 7). The specimens which were of a metallic colour are marked distinctively on the graph. Their temperatures did not differ much from the average, so variation in temperature arising from colours between metallic blues and greens, dull browns and black is very slight.

This, however, gives us no idea of absorptivity, although we may expect that a dark-coloured insect is not far from being a black body. Another way of assessing the effect of colour on temperature excess is to find its temperature excess when normal and again when painted black, to convert it to a black body (absorptivity 100%) of the same size and shape. In a series of experiments, after obtaining the temperature excess of an insect as usual, the dorsal surface was painted with black oil paint or with an aqueous suspension of lamp-black and the temperature excess found again, after a suitable period to allow evaporation of the solvent. The results are given in Table 3.

The most notable point is that a number of insects were hotter (in the middle of the body) in the normal state than when painted black. As the insect absorbs the maximum amount of heat when painted black it cannot be true to say that the temperature excess varies with absorptivity. It seems better to refer to the proportion between the temperatures as apparent absorptivity. These apparent absorptivities for the normal insects ranged between 63 and 117%. Pale forms, green to dark yellow, ranged from 63 to 86%; dark forms, browns, blacks and metallic greens, blues and blacks, from 71 to 117%.

The explanation for this apparent absorptivity in excess of 100% probably lies in the site of absorption. Heat produced is carried away by conduction and convection to the air, and by conduction to the underlying body of the insect and to the other cooling surfaces (radiation being very slight). Where the surface is highly

absorbing, the heat is produced at the surface where it will readily be carried away; but where the surface absorbs little of the heat, more radiation will be available for absorption throughout the thickness of the thorax. In this case, as cooling is only at the outer surface, the inside will be hotter than the outside. Such an effect is known in other fields—for instance in the melting of snow beneath the surface at low air temperatures.

Table 3. *Temperature excess* (° C.) *of various insects as normal and when painted, subjected to wind of* 50 *cm./sec. and to radiation from bulb*

Insect	Colour	Temperature excess (° C.)		Apparent absorptivity (%)		
		As normal	Thorax painted black	When normal	When painted	
					Green	White
Carausius morosus (Orthop. Phasmidae)	Green*	7·6	12·0*	63	—	—
C. morosus (Orthop. Phasmidae)	Green	1·54	2·20	70	—	80
Periplaneta americana (Orthop. Blattidae) nymph	Brown	3·23	4·55	71	—	64
Schistocerca gregaria (Orthop. Acrididae) adult	Dull yellow-green*	10·1	13·4*	75	—	—
Periplaneta americana (Orthop. Blattidae) nymph	Brown	2·4	3·2†	75	—	—
Calliphora erythrocephala (Dipt. Tachinidae)	Dull blue-black	1·43	1·87	77	88	80
Vespa vulgaris (Hym. Vespidae) abdomen	Yellow and black	4·2	5·3†	79	—	—
Schistocerca gregaria (Orthop. Acrididae) stage V hopper	Dull yellow-green	2·0	2·5†	80	—	—
S. gregaria (Orthop. Acrididae) stage V hopper	Dark yellow	1·76	2·2	80	—	—
S. gregaria (Orthop. Acrididae) stage IV hopper	Dark yellow	2·90	3·36	86	94	72
Lucilia caesar (Dipt. Tachinidae)	Metallic green	2·4	2·4†	100	—	—
Phormia caerulia (Dipt. Tachinidae)	Metallic blue-black*	8·2	7·9	104	97	85
Phormia caerulia (Dipt. Tachinidae)	Metallic blue-black*	3·0	2·8†	108	—	—
Eristalis tenax (Dipt. Syrphidae)	Brown	3·5	3·2†	109	—	—
Sarcophaga carnaria (Dipt. Tachinidae)	Dull blue-black	2·8	2·5†	112	—	—
Mesembrina meridiana (Dipt. Tachinidae)	Black	3·5	3·0†	117	—	—

* Experiments run at 1·5 cal./cm.²/min. with water filter; otherwise run at 0·5 cal. with copper chloride filter to give correct spectral composition.

† Specimens painted with aqueous suspension of lamp-black; otherwise artists' oil paints used.

That the painting of an insect affects the distribution of temperature is shown in Table 2 for the stick insect and the locust. The normal stick insect was a little cooler on the upper surface than a little way inside the thorax; in the normal locust the temperatures were equal. Blackening caused a great increase in the surface temperature and in this case a much smaller increase in the internal temperatures. It is,

moreover, interesting to note that the lower surface of *Carausius*, both normal and when blackened, intercepted enough of the radiation which had passed through the thickness of the body to make the ventral surface hotter than the inside.

The range of effect to be expected in colour alone was investigated by painting some of the specimens green or white. For although the pigments in paint are not those found in the insect, the range of temperatures of normal insects of various colours should be fairly similar to that of a series of painted insects, in so far that differences of temperature are due to differences in colour in the visible alone. The apparent absorptivities of green-painted specimens lay between 88 and 97%, of white, 64 and 85%. This high absorptivity of the light-green or white-painted insect arises from the fact that, as Gunn (1942) has pointed out, a large part of the radiation is in the infra-red, of which the white paint (titanium white) absorbs a large part. As half the radiation is in the infra-red, temperature bears as much relation to absorption in the infra-red as to absorption in the visible.

It may be noted that roughly similar results were obtained by Dorno (1931) (quoted by Geiger, 1950) for painted wooden posts heated by sunshine. The temperature of a white-painted post reached 64% of that of a black post. These experiments have given results which are in reasonable agreement with the absorptivity measurements of Rucker (1933*a*, *b*, 1934) and Duspiva & Cerny (1934). From their measurements, if we say that in extreme cases insects which differ only in colour will have a constant absorption of say 50% in the infra-red and an absorption in the visible which varies from 26 to 95%, then the lightest insect, a chalky white form, will be expected to achieve 52% of the temperature excess of the darkest; insects of the more usual light colours (that is, blues, greens, yellows) will be expected to achieve 75% of the temperature excess of the dark forms. It is, however, noticeable that in many light-coloured insects the thorax itself is dark. Temperature differences due to variation in colour will therefore be of minor importance.

It is convenient to consider pubescence at this point. In the experiments run for the effect of size on temperature (Fig. 7), pubescent specimens appear to be no warmer than their non-pubescent neighbours. This is perhaps surprising, but as the experiments were run at a wind speed of 50 cm./sec., quite possibly the effects of pubescence are only of significance at lower wind speeds where viscous forces in the air flow come more into play.

(iv) *Size*

The curves of the increase of temperature against time obtained for six insects at a wind speed of 50 cm./sec., when a radiation strength of 1·5 cal./cm.^{2}/min. was switched on, are shown in Fig. 6. The larger insects attained the higher temperatures and took longer to attain them.

The relation of equilibrium temperature excess to size for insects (Table 4) and for spheres of black plasticine at the same radiation and wind speed is shown in Fig. 7 as a log-log plot. The size measured was the diameter in the case of the plasticine spheres and the maximum breadth of the thorax close to the base of the wings in the case of the insects. In the plasticine spheres the temperature excess varied as a power of the size between 0·7 for spheres below 0·3 cm., and 0·4 for

larger sizes (0·3–2·0 cm.). Size for size, temperatures of the locusts varied between the temperature of the spheres and twice this value. Most of the points for these insects are ranged about a line indicating that temperature excess varies as about the 0·4 power of the size. Temperatures of the Diptera and Hymenoptera were from half as much again to three times as great as those of the spheres, varying approximately as the 1·0 power of the size.

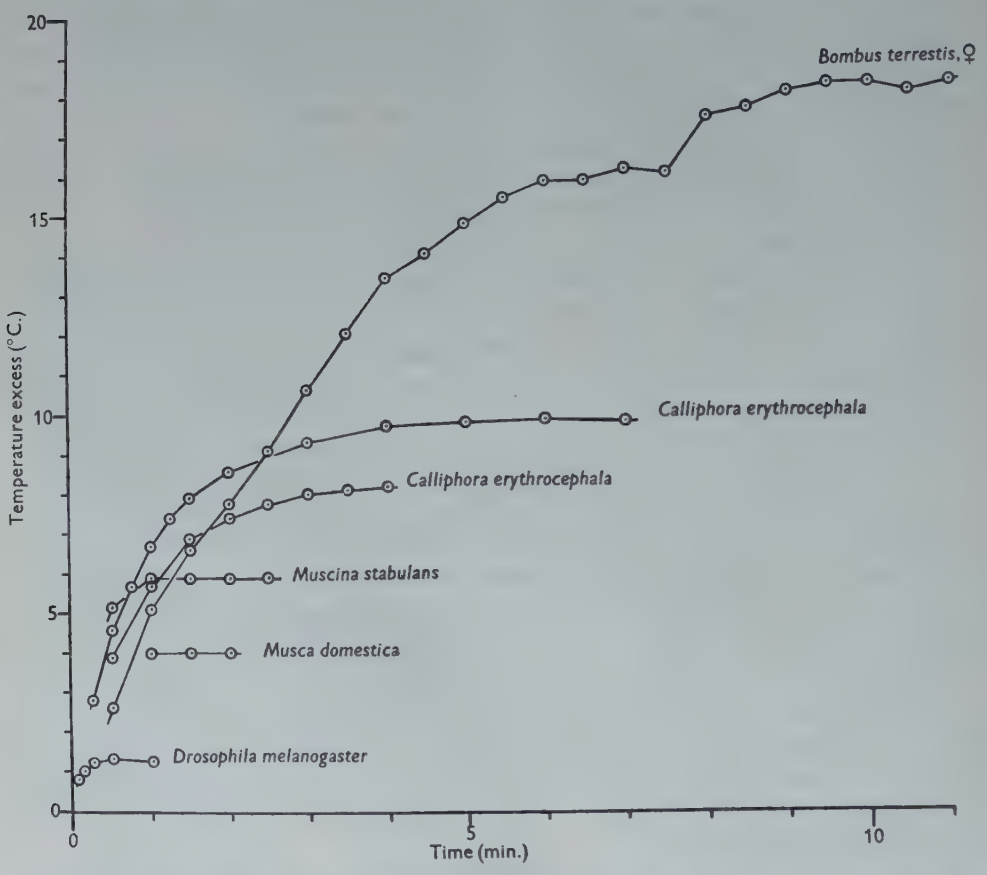

Fig. 6. Increase in temperature excess in various insects when radiation of 1·5 cal./cm.²/min. switched on. Wind 50 cm./sec. In *Bombus* departures from smooth curve coincide with slight movements of thorax, wings and legs.

Temperature may be expected to vary with the 0·5 to 0·6 power of the size when conditions of forced convection prevail, but when velocity or size becomes very small this power may be expected to increase to 1·0. The plasticine spheres agree reasonably well with theory, but the insects, particularly the Diptera and Hymenoptera, show a much steeper slope than is expected. Why is the slope so steep? Transition to the 1·0 power of the size should only occur in much smaller specimens (i.e. when *Nu* approaches 2, see Appendix.) The possibility of part of the effect being the loss of

heat via the thermocouple wires in the smallest forms of about 0·3 cm. diameter cannot be entirely ruled out. Natural convection effects cannot be the cause because a tendency to natural convection only results in a reduction of temperature of the very largest specimens at this wind speed; and this would cause the curve to be less steep; to tend towards the 0·2 power. The most likely explanation is that the steepness is due to change in absorptivity or the position of absorption (perhaps both) with size.

Table 4. *Relation of temperature excess to size in a series of insects of squat body form (Diptera-Hymenoptera type) and of elongate body form (locust type), at a radiation strength of 1·5 cal./cm.²/min. and a wind speed of 50 cm./sec.*

Breadth of thorax (cm.)	Diptera-Hymenoptera type, species	Mean temp. (° C.)	Locust type, species	Mean temp. (° C.)
1·5–2·5	*Sphaerophoria scripta* (Dipt. Syrphidae), 3, 1 *Syritta pipiens* (Dipt. Syrphidae), 2 *Musca domestica* (Dipt. Anthomyidae) *Eristalis arbustorum* (Dipt. Syrphidae)	5·2	*Schistocerca gregaria* (Orthop. Acrididae), stage I hopper, 2	4·5
2·6–3·5	*Lucilia caesar* (Dipt. Tachinidae), 2 *Sarcophaga carnaria* (Dipt. Tachinidae), 2 *Phormia caerulia* (Dipt. Tachinidae), 3 *Eristalis arbustorum* (Dipt. Syrphidae) *Apis mellifica* (Hym. Apidae) *Vespa vulgaris* (Hym. Vespidae)	8·9	*Carausius morosus* (Orthop. Phasmidae) *Schistocerca gregaria* (Orthop. Acrididae), stage II hopper	6·7
3·6–4·5	*Calliphora erythrocephala* (Dipt. Tachinidae), 2 *Echinomya fera* (Dipt. Tachinidae) *Apis mellifica* (Hym. Apidae) *Psithyrus campestris* (Hym. Bombidae) *Eristalis nemorum* (Dipt. Syrphidae) *Mesembrina meridiana* (Dipt. Tachinidae) *Eristalis tenax* (Dipt. Syrphidae), 3 *Bombus terristis* w. (Hym. Bombidae)	9·6	*Carausius morosus* (Orthop. Phasmidae) *Schistocerca gregaria* (Orthop. Acrididae), stage III hopper, 2	6·2
4·6–6·5	*Psithyrus campestris* (Hym. Bombidae)	8·0	*S. gregaria* (Orthop. Acrididae), stage IV hopper, 3	7·9
6·6–8·5	*Bombus lapidarius* w. (Hym. Bombidae)	12·0	*S. gregaria* (Orthop. Acrididae), stage V hopper, 4	6·4
8·6–10·5	—	—	*S. gregaria* (Orthop. Acrididae), adult, 2 *Periplaneta americana* (Orthop. Blattidae), nymph, 2	9·6

(v) *Wind*

Curves relating temperature excess to wind speed at a radiation strength of 1·5 cal./cm.²/min. are given for two plasticine spheres and for a number of insects of varying size as a log-log plot in Fig. 8. In both insects and spheres temperature varied inversely as the square root of the wind velocity when the latter was above 20–30 cm./sec., but below these wind speeds the slope decreased towards the point (not reached) where temperature would be independent of wind velocity. This

indicates that above 20–30 cm./sec., forced convection was occurring, but below these speeds conditions of natural convection with temperature independent of wind speed were approached. Larger specimens showed an approach to natural convection at a higher wind speed than did smaller specimens because of their greater temperatures.

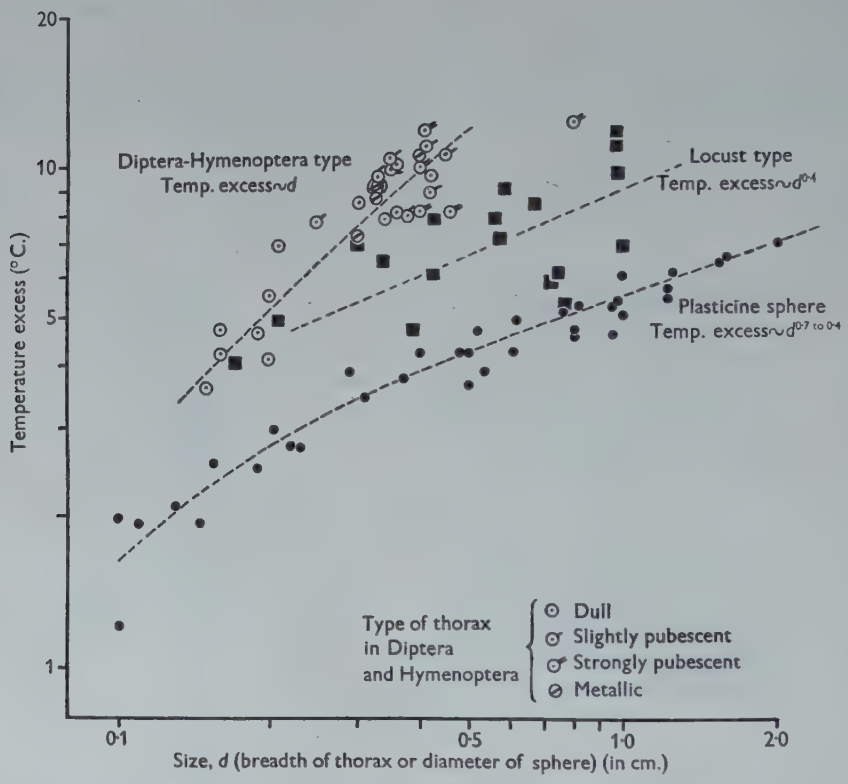

Fig. 7. Variation in temperature excess with size for a series of insects and plasticine spheres, log-log plot. The slope of the curves changes with size, the temperature excess varying with a power of the size between 1·0 and 0·37. Radiation 1·5 cal./cm.²/min., wind 50 cm./sec.

There is a certain amount of variation in temperature according to the orientation of the insect. The above experiments were all performed with the thermocouple in the thorax and the insect facing into the wind. Variation in temperature excess according to orientation to wind (orientation to radiation being constant) is shown for five insects in Table 5. There was practically no difference in *Periplaneta*, a squat form, but in *Carausius*, of elongate cylindrical shape, the temperature was reduced by nearly one half by turning sideways on. Thus an elongate insect may well exercise some control of its body temperature by orientation to wind. This is to be expected, for it is known that heat loss from a cylinder with air flow normal to the long axis is about twice that with the air flow in the direction of the long axis.

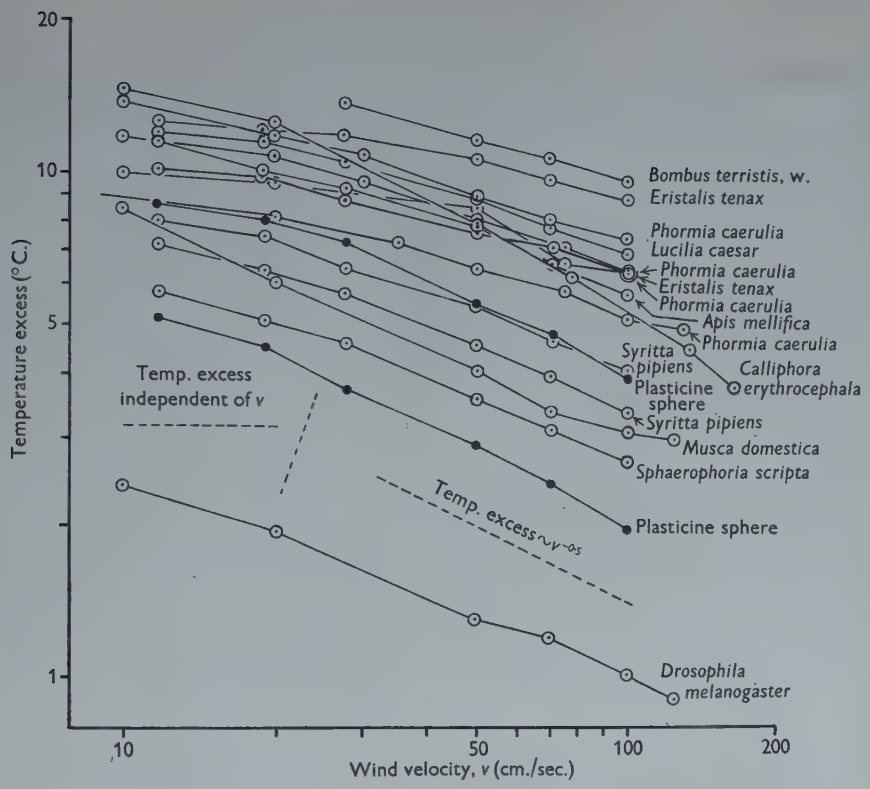

Fig. 8. Variation in temperature excess with wind velocity, log-log plot. Each line represents a run for a single insect or plasticine sphere. The slope of the curves changes with wind velocity, temperature excess varying inversely with the square root of the wind speed at high speeds, but tending to be independent of wind at low speeds (transition between natural and forced convection). Radiation 1·5 cal./cm.²/min., wind 5–150 cm./sec.

Table 5. *Variation in temperature excess with orientation to wind for insects of differing shape*

Species	Proportion length/breadth of insect	Temp. facing air stream (as usual)	Temp. excess at 45° to wind, as percentage of temp. excess facing wind (%)	Temp. excess at 90° to wind, as percentage of temp. excess facing wind (%)
Periplaneta americana (Orthop. Blattidae), nymph	2·5 : 1	3·16	99	99
Calliphora erythrocephala (Dipt. Tachinidae)	2·6 : 1	1·54	100	97
Schistocerca gregaria (Orthop. Acrididae), stage IV hopper	5·6 : 1	2·99	98	88
Schistocerca gregaria (Orthop. Acrididae), stage V hopper	5·4 : 1	1·76	—	86
Carausius morosus (Orthop. Phasmidae)	15·1 : 1	1·58	67	56

(vi) *Evaporation and metabolism*

Specimens of *Calliphora*, *Phormia*, *Muscina* and *Schistocerca* when mounted and subjected to wind speeds of 10–100 cm./sec. and no radiation approximated to the temperature of the air stream to 0·05° C. or closer, the temperature drop from evaporation being practically negligible. This is to be expected, as the water loss is slight.

It is well known that the metabolic activity of flight results in a substantial rise in temperature in large insects. A similar smaller rise occurs in these smaller forms. When a live fly was mounted with a thermocouple in the thorax, flight quite often appeared to be normal in the rate of wing beat and duration; when flying began in a mounted specimen of *Calliphora* with no radiation and a wind speed of 50 cm./sec. the temperature typically showed a momentary drop of about 0·1° C., then a rise of up to 2·0° C. in 1 or 2 min. Most of the rise occurred in the first 30 sec., and it was followed by a slow drop of 1·0° C. after flight had continued for some minutes. This temperature rise from the metabolic activity of flight was found to be dependent upon wind speed as was the temperature rise from radiation; thus with *Muscina* flying at wind speeds of 0, 50 and 100 cm./sec., with no radiation, the temperatures attained were 1·3, 1·1 and 0·8° C. above that of the air, respectively. Occasionally the temperature of a mounted but apparently inactive insect could be seen to rise; in the case of *Calliphora*, to 0·75° C. above that of the air (with wind at 50 cm./sec. and no radiation); the same phenomenon was observed by Krogh & Zeuthen (1941) in various larger insects. Sometimes this led to flight, but sometimes the temperature dropped again to that of the air. With this form of activity, the temperature never rose to more than 0·8° C. above that of the air, as against some 1·5° C. for full flight under the same conditions. This warming was not preceded by the momentary drop in temperature that marked the initiation of flight. The momentary drop is probably due to internal ventilation and evaporation.

This metabolic heating might be expected to result in an insect becoming hotter when flying in sunshine than when basking, other things being equal. But such is not the case. When flight began during exposure to radiation of 1·5 cal./cm.^{2}/min. the temperatures of *Calliphora* and *Muscina* which had become steady at 4–6° C. above that of the air, showed only a slight change. This ranged between a decrease of 0·5° C. and an increase of 0·3° C., notwithstanding the fact that in the absence of radiation flight would cause a rise of 1·5–2·0° C. This indicates that the extra heat input was counterbalanced by the extra loss from internal ventilation and evaporation. It follows that similar temperatures attained by flight activity and by radiation do not represent similar rates of heat in-put.

The heating effect of metabolism becomes less important compared with that of the sun as size decreases. Thus while an insect the size of a hawk moth would appear to attain a roughly similar temperature excess by metabolism as it would in strong sunshine, a small fly (*Muscina*) will only attain by metabolism about a fifth of its own sunshine temperature excess. Metabolic heating will decrease with the cube of the linear dimension, radiative heating with the square.

DISCUSSION

The position of long-wave radiation has been neglected so far. An insect is probably usually cooler than its surroundings, for in both natural and forced convection the rate of heat loss is greater from a small isolated body than from a flat surface. An insect will therefore gain heat by radiation from the background and this gain will be greatest with a dry background, for moist vegetation is kept cool by evaporation. As a probably exceptional case, an insect at 20° C. surrounded by a hemispherical background at 40° C. can be shown to gain heat at about 0·1 cal./cm.²/min. Such a temperature difference of 20° C. would only be maintained in bright sunshine with direct plus reflected short-wave radiation of at least 1·5 cal./cm.²/min. Over the small range of temperature considered, radiation is nearly proportional to temperature difference, and a more likely temperature difference of 10° C. would result in a long-wave radiation contribution of only some 4% of the total.

The main factors controlling the temperature excess for small insects are radiation, wind and size. For insects of the Diptera-Hymenoptera type resting in a position where they were subjected to a 50 cm./sec. wind and strong sunshine from above, the temperature excess would be: *Drosophila* (1·5 mm. broad), 1·5° C.; *Syritta pipiens* (2 mm. broad), 5° C.; *Calliphora* (4 mm. broad), 8° C.; *Bombus* (8 mm. broad), 12° C. Insects similar to the locust would be half to two-thirds as hot, size for size. These figures hold for bright sunshine in spring (1·5 cal./cm.²/min.). Later in the year the sunshine is commonly weaker (1·0 cal./cm.²/min.) and the temperature will be two-thirds of the above. A reasonable value for sunshine just visible through cloud is 0·25 cal./cm.²/min.; in this case the temperature will be one-sixth that of the above. If the wind dropped from 50 to 10 cm./sec. the temperature would be doubled in the case of the smaller insects but would only be half as much again in the larger forms. Still lower winds result in still higher temperatures—possibly half as much again as the 10 cm./sec. values; we do not know the limit for the maximum temperatures attainable under true natural convection. A very small alteration in microclimatic wind at these low speeds can cause great differences in temperature. A limited amount of temperature control is possible by orientation to wind, but this method is only open to insects of the locust type. Colour has little effect, not more than some 25% as a rule; most insects, however, have a dark thorax.

Calculation of the temperature of insects from the relations which are known to exist between temperature excess, size and wind speed suffer from the disadvantage that the convection conditions which prevail close to the ground lie between those of natural and forced convection, where the expressions appropriate to either do not hold good. This region could, however, be covered by empirical relations obtained by the manner employed in this paper. The application of such observations to the field is at present limited by the lack of field observations on microclimate, particularly wind speed and temperature close to the ground.

It seems probable that to attain as high a temperature excess as possible for a given size is of selective significance, for insects are so dependent on temperature for their activity. This suggestion is supported by the way in which the thorax of an

insect would appear to be adapted to that end. The thorax of an insect may be regarded as a sphere protected from convective heat loss by the head in front and the abdomen behind. The heat loss from a sphere is greatest on the sides towards and away from the air flow and least at the sides (see Bairstow, 1939). The Diptera and Hymenoptera, which on morphological grounds include the most highly developed forms, show a much greater temperature excess than the locust, size for size, and in these the head and abdomen would appear to be attached to the thorax by as slender supports as possible, thus minimizing conduction. The layer of air between the head and thorax, and thorax and abdomen is probably kept stagnant by pubescence; conductivity through such a layer of air is very small. The colour of the thorax is usually dark, and sometimes the structure is such that the apparent absorptivity can be in excess of 100 %. The outer surfaces of the thorax are frequently covered with bristles or pubescence, which although it does not apparently increase the temperature excess in the small range of forms studied at a wind speed of 50 cm./sec., it may well be that it has an important effect at very low speeds where viscous forces in the air stream come more into play. This is highly suggestive that the body form of the most highly developed types has evolved in response to the necessity for obtaining the maximum warmth from the sun.

SUMMARY

1. The temperature excess developed by insects under known radiation strength equivalent to sunshine has been studied in relation to modifying factors in a wind tunnel in the laboratory.

2. Temperature excess was measured by a thermocouple unit in the larger insects, and by the increase in rate of wing beat in *Drosophila*.

3. Temperature excess varied directly with radiation strength.

4. For insects of breadth greater than 0·3 cm. spectral composition of radiation over the normal sunshine range was of negligible importance to the temperature excess.

5. The effect of colour on the temperature excess was slight. Absorptivity was estimated by comparing temperatures of normal and black-painted insects. Some insects were hotter in the normal state because of change in the site of absorption of heat. White-painted insects were 64–85 % as hot as when black painted, because the white paint still absorbs infra-red radiation.

6. Temperature excess varied with a power of the size between 0·7 and 0·4 in the plasticine spheres, according to size. Temperature excess of the locust type varied as about the 0·4 power of the size; of the Diptera-Hymenoptera type, as about the 1·0 power. Size for size, compared with the spheres, the locust became between half as hot again and twice as hot, and the Diptera-Hymenoptera type between half as hot again and three times as hot, as the plasticine spheres.

7. Temperature excess varied inversely as the square root of the wind speed above speeds of 20–30 cm./sec. and tended to become independent at lower speeds, with the transition between forced and natural convection. The latter condition will be more important close to the ground.

8. Temperature rise by flight activity is not additive to temperature rise from radiation because it is associated with extra cooling.

9. Evolution of insects from the type of the Orthoptera to the Diptera and Hymenoptera has been associated with the attainment of greater temperature excess for given body size. This is probably of selective significance.

This work was carried out at the Department of Zoology and Comparative Anatomy at Oxford, supported by an Agricultural Research Council grant, and in the Department of Biology, St Thomas's Hospital Medical School, London. The wind tunnel used was originally constructed at the School of Agriculture, Cambridge. For facilities and encouragement I am grateful to Prof. A. C. Hardy, F.R.S., Dr A. G. Hamilton, Mr F. R. Petherbridge, Mr J. H. Stapley and Mr D. W. Wright. I am indebted to Dr G. D. Robinson for the loan of a Michelson actinometer and to Dr W. Cockrane and Dr M. Fishenden for helpful discussion. I am grateful to Prof. A. C. Hardy, F.R.S., for reading the manuscript and to my wife for her help at many stages.

APPENDIX

The relationships for the convective heat loss from cylinders and spheres, as given by Fishenden & Saunders (1950) are:

(i) In the absence of applied air flow (natural convection—here considered in the streamline range), for cylinders,

$$\frac{H}{\theta} = 0{\cdot}24\left(\frac{\theta}{d}\right)^{0{\cdot}25},$$

where H = heat transfer per unit area, unit time; θ = temperature excess at surface; d = diameter. No data is available for spheres.

(ii) In the presence of an applied air flow (forced convection), with air moving across a cylinder, for Reynolds number (Re) 1000–100,000

$$Nu = 0{\cdot}24\ Re^{0{\cdot}6},$$

where Nu = Nusselt number, $= Hd/k\theta$; $Re = v\rho l/\mu$; v = velocity of air; k = conductivity of the air; ρ = density of the air; μ = viscosity of the air.

In the range Re = 10–200 covered in this paper, the relation $Nu = 0{\cdot}7\ Re^{0{\cdot}4}$ might be expected to be more appropriate.

With air moving along a cylinder, which is more comparable to the case of an insect facing the air stream, heat flow is half this value. For a sphere the formula is the same apart from the constant. When v is small, Nu reaches a limiting value of 2; hence,

$$\frac{H}{\theta} = \frac{2k}{d}.$$

Now the factor in which we are most interested is the temperature excess of the inside of the thorax. In engineering problems the factor which is of prime importance is the heat transfer coefficient, H/θ. In these experiments, as the heat has been applied by radiation and we do not know the true absorptivity, we do not know the

rate of heat gain, H. Neither do we know the true mean surface temperature excess, (θ) although it is probable that it is not very different from the temperature of the inside of the thorax. We are therefore not in a position to calculate exactly either the heat transfer coefficient or the Nusselt number. It is therefore most suitable to consider simply the temperature excess in relation to the other terms, i.e. radiation, size and wind speed, etc.

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OBSERVATIONS ON LUMINESCENCE IN *RENILLA* (PENNATULACEA)

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Luminescence is well known in three groups of coelenterates, viz. Hydrozoa, Scyphozoa and Alcyonaria (literature reviewed by Harvey, 1952). Of these *Pelagia*, *Pennatula* and *Renilla* have been most intensively investigated. Observations indicate that in all these forms, light emission is under nervous control. Mechanical and electrical stimulation gives rise to a wave of light which passes over the surface of the animal (the disk in medusae, or the surface of the colony in hydroids and alcyonarians), at a rate which is consonant with nervous transmission as deduced from the spread of excitation concerned with muscular contraction. In his pioneering studies of the physiology of luminescence, Panceri (1872*a*, *b*) found that localized stimulation of *Pennatula* excites luminous waves which spread up and down the colony, according to the position of excitation. Simultaneous excitation of the two extremities of an animal gives rise to two converging waves which cancel out on meeting. Parker's (1920*b*) detailed study of *Renilla*, which will be referred to later, showed that luminescence is controlled by a nerve net, and that cuts made in any direction through the rachis still permit excitation to be transmitted so long as the stimulated region is not completely isolated, i.e. the nerve net is non-polarized.

In a recent study at the Kerckhoff Marine Laboratory, Buck (1953), employing visual observation, investigated neural regulation of luminescence in *Renilla*, and concluded that the siphonozooids emit brief flashes, which make up the luminous wave sweeping over the colony. Conduction was found to be non-polarized and non-decremental, and facilitation was clearly shown. The present investigation of *Renilla*, in which electronic recording has been employed, confirms and amplifies previous observations, and is intended to form a background for more detailed studies.

MATERIAL AND METHODS

This investigation was carried out at the Scripps Institute of Oceanography, La Jolla, California. Specimens of *Renilla köllikeri* (the sea pansy) were collected from the littoral region, dredged, or secured by divers from shallow sublittoral waters. They were kept in aquaria under running sea water until required for experimentation.

Experiments were carried out in a darkened room. The animal was set on a platform containing a hole to accommodate the peduncle, and placed in a vessel

* Guggenheim Canadian Fellow, 1953.

containing running sea water. The water temperature varied from 15 to 22° C., owing to fluctuation of supply, but an attempt was made to hold the temperature to within a few degrees during the course of any one experiment; in any case, the temperature variation is noted. Visual observations were supplemented by electronic recording. Stimulation was mechanical and electrical; for the latter a relaxation oscillator was used to deliver condenser discharges. Half-decay time for condenser pulses was 20 msec. Electrodes were non-polarizable Ag/AgCl or Pt/Pt black.

Luminescence was recorded with an E.M.I. multiplier phototube (Type 6685), d.c. amplifier, and cathode-ray oscilloscope. Total delay in this system was of the order of a few microseconds. The spectral response curve of the photocathode ranges from 300 to 600 mμ, with a peak at about 420 mμ. Spectral characteristics of the light of *Renilla* have not been determined, but with a hand spectroscope light was found confined to the blue end of the spectrum. The emission band of luminescent Hydromedusae is known to extend from *c.* 460–600 mμ (Harvey, 1952). Amplification was regulated by varying voltage on the dynodes and by controlling amplifier gain. Since there is enormous variation in intensity of light emission in different specimens, at different times in the same specimen, and under different conditions of stimulation, it was necessary to change frequently the amplification of the recording apparatus. For purposes of publication some of the response curves have been redrawn and converted to a common scale. Other details of procedure are noted in the text.

STRUCTURE AND HABITS OF *RENILLA*

The sea pansy *Renilla* is a warm-water New World form. *R. köllikeri* occurs from Santa Barbara* to Cedros Island (Lower California) where annual water temperatures range round 13–22° C. (Ricketts & Calvin, 1952; Ekman, 1953). It is found on intertidal sand-mud flats, and extends down to 20 m. or so. It has a heart-shaped disk or rachis, up to 10 cm. in diameter; the upper surface of the rachis bears polyps, and from the under surface a fleshy peduncle extends downward. Parker (1919, 1920*a*, *b*) and Buck (1954) have described the structure and habits of *Renilla* in some detail. Of immediate interest is the observation that mechanical and electrical stimulation causes contraction of the body and withdrawal of the polyps. Luminescence is confined to the upper surface of the rachis (Parker, 1919).

Two modes of luminescence can be distinguished in *Renilla*. The most obvious is progressive flashing which takes the form of waves sweeping over the surface of the colony from the point of stimulation. Each wave front results from the temporary glowing of many luminescent points, which are excited successively as the wave courses over the surface. In addition, discrete points can be described in which excitation evokes a prolonged slowly decaying glow. The luminescence is localized in the surrounds of the siphonozooids, in the calices at the bases of the autozooids, and in the autozooids themselves. According to Buck luminescence is intracellular.

* Personal observation of E. Noble and D. Davenport.

The siphonozooids emit the brief synchronized flashes, while the autozooids are responsible for the more prolonged glow (Parker, 1920*b*; Buck, 1953, 1954). Since the autozooids usually retreat under stimulation, most of the observations to be described refer to the responses of luminescent tissue around the bases of autozooids and siphonozooids.

GENERAL OBSERVATIONS ON THE LUMINESCENT RESPONSE

In an animal which has been dark-exposed for some time, luminescence is evoked by tactile or electrical stimulation. Following tactile stimulation, luminescent waves arise in the affected region and run over the animal. As noted by Buck (1954), the individual waves thus induced succeed each other rapidly at first, but soon decrease in intensity and frequency. A strong protracted stimulus, such as strongly pressing the animal, gives rise to a bright even glow affecting the whole rachis. With continued mechanical stimulation, some form of adaptation or accommodation frequently occurs; luminescent waves become less frequent, even to strong stimuli, and soon cease; and the response then consists of localized brightly glowing points in the area

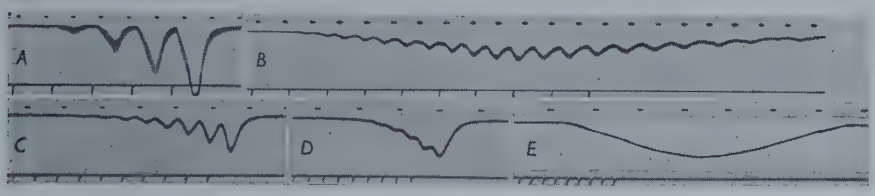

Fig. 1. Luminescent responses of *Renilla* to electrical stimulation. Recordings from the whole animal. Time signal above each record, 72 per min. Downward deflexions of middle trace are luminescent responses. Electrical stimuli (condenser shocks) shown on lower line. *A*, burst of 5 shocks at 1 per sec., response appears on the 2nd shock, and subsequent flashes increase in intensity. *B*, rhythmic after-discharge induced by a burst of 10 shocks at 1 per sec. *C*, burst at 2 per sec. *D*, 3 per sec. *E*, 5 per sec. Records from several specimens at different amplifications. Temp. *A*, 25° C.; *B*, 17° C.; *C–E*, 19–22° C.

of stimulation. Panceri (1872*a*) records the same effect after repeated experimentation in *Pennatula*. The onset of refractoriness of this kind is certainly not peculiar to the luminescent response, and resides in the excitatory system, since Pantin (1935*a*) noted a similar effect when investigating muscular responses of sea anemones.

With electrical stimulation, luminescent waves are evoked, which arise under the electrodes and travel across the surface of the rachis (Fig. 1). At normal temperatures (15–17° C.), close to those at which the animal has been acclimatized, several shocks are required to elicit a response. The quantitative relations depend on the frequency of stimulation. At a frequency of 1 per sec. or more, the first overt response occurs normally on the 3rd stimulus, sometimes on the 2nd, and a response accompanies each subsequent stimulus (Fig. 1, *A*, *B*, *C*). At slower frequencies more stimuli are usually necessary to elicit the first response (Fig. 2). Threshold

frequency lies around 12 per min. (interval 5 sec.), at which rate the individual responses are very weak. The following is a protocol of a typical experiment:

Frequency	First effective stimulus	Subsequent flashes	Remarks
20 per min.	16	On 21, 27, 33, 36, 40, 42, 45, 49, 51, 53rd shock and each subsequent stimulus	Very faint flashes
30 per min.	9	1 per shock thereafter	Very faint flashes
42 per min.	4	1 per shock thereafter	2nd and subsequent flashes brighter
60 per min.	3	1 per shock thereafter	Subsequent flashes increasing in intensity
120 per min.	2	I per shock thereafter	Bright flashes
180 per min.	3	1 flash per stimulus up to 15th shock, and a flash for every 2nd or 3rd subsequent stimulus	Bright flashes

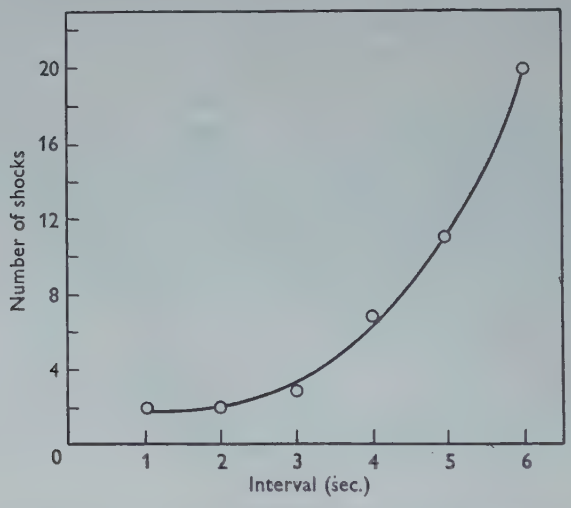

Fig. 2. Relation between stimulus interval and number of stimuli required to elicit the first photogenic response. Data from one specimen (17·1° C.). Other animals gave similar curves, but differing in slope.

With slow frequencies (intervals 3 sec. or more), the first response is observed after 10–16 shocks, varying with the specimen. Responses thereafter may occur on each stimulus, or may be spaced at longer intervals, with a flash on each 5th or 6th stimulus. With continued stimulation the response-rate increases, and finally comes into correspondence with stimulation-frequency, one wave following each shock.

At higher frequency (*c.* 3/sec.) the responses at first follow the stimuli faithfully, 1 flash per shock, but after 10–15 stimuli, the flashes cease to appear at the rate of stimulation, and a light-wave arises on each 2nd or 3rd shock. At still faster rates (up to 7 per sec.) only every 2nd or 3rd shock is effective *ab initio*.

Continued flashing often occurs after stimulation has ceased. This post-stimulatory flashing is observed after bursts of stimuli at frequencies of 1 per sec. or more

(Figs. 1, *B*; 3, *C*). After a burst of 10–15 shocks, continued flashing may develop at a rate of 1 per sec. and persist for 10 sec. or longer. During this post-stimulatory flashing, the frequency falls off and intensity progressively decreases. With long-continued stimulation Buck (1954) describes how the animal passes into a hyper-excitatory state in which flashing continues for very long periods (30–60 min.). In this condition multiple waves arise from many loci so that the whole surface appears

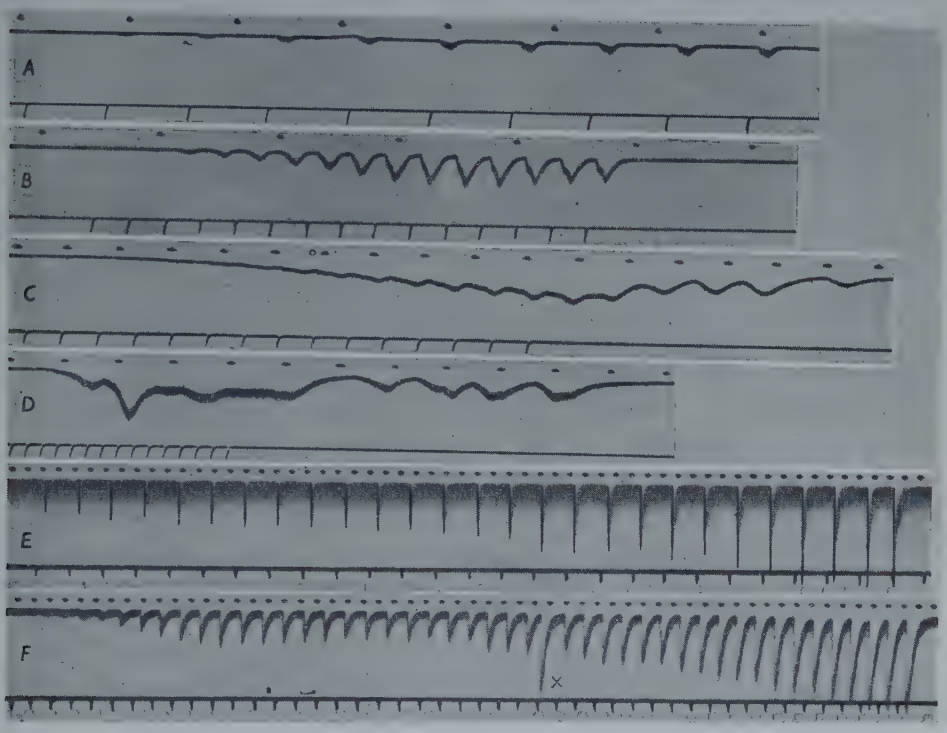

Fig. 3. Luminescent responses in *Renilla*, produced by electrical stimulation at different frequencies. *A–D*, recordings from the whole animal. *A*, 21 per min., amplification × 100. *B*, 60 per min., amplification, × 30. *C*, 120 per min., amplification, × 10. *D*, 240 per min., amplification, × 30. *E*, *F*, luminous response to prolonged repetitive electrical stimulation. *E*, burst at 42 per min. The first 5 stimuli have been removed from this record, and response first appears clearly above background on the 6th shock. *F*, another burst at 1 per sec. The bright response marked at × in this record is the result of an induced intercalated impulse interacting with an external stimulus. Slit recordings (slit aperture 5 × 15 mm. with long axis parallel to wave front). Time scales, *A*, *B*, 14 per min.; *C–F*, 72 per min. Temp. 17°.

rippling with light. As this hyper-excitatory state decays the waves decrease in intensity but may continue to display multiple centres of origin; or excitability may become focused at one point, from which waves arise with persistent regularity.

The intensity of response is influenced by stimulation frequency and number of stimuli. At slow rates of stimulation (intervals of 1 sec. or more), each photogenic response returns to zero before another arises. Under these conditions it is observed that responses subsequent to the first increase progressively in intensity (Fig. 4).

Since summation of overt responses is here excluded, the resultant build-up of intensity can be ascribed to facilitation. At a frequency of around 1 per sec., the increment of intensity of consecutive responses increases rapidly after 4–5 shocks, and gradually rises to maximal plateau level. The number of responses ensuing before plateau is reached varies in different specimens, but usually lies between 12 and 30 at frequencies of 30–60 per min. Thereafter the response intensity may hover steadily at plateau level for some time (Fig. 4, *B*). Frequently, however, there is subsequent decline in the intensity of consecutive responses once plateau or maximum is reached, especially at frequencies above 1 per sec. (Figs. 3, *B*; 4, *A*).

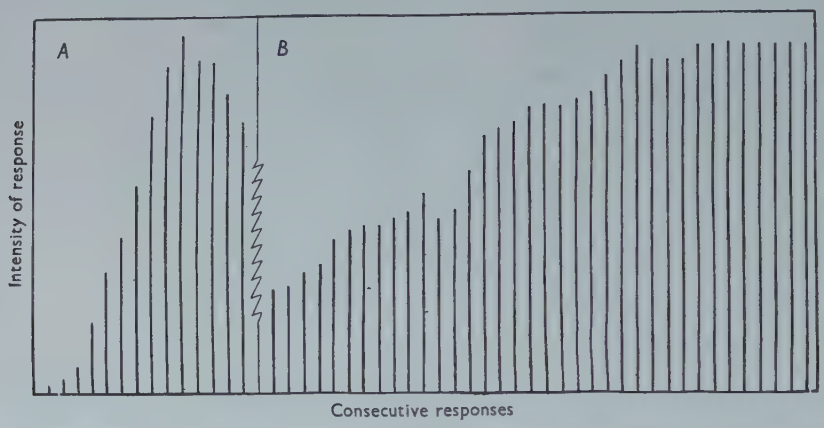

Fig. 4. Intensities of consecutive responses evoked by electrical stimulation. *A*, response to a burst of 14 shocks at a frequency of 1 per sec. First response on 2nd stimulus. *B*, responses to a burst of 42 shocks at a frequency of 42 per min. Response appeared on 3rd shock, but measurable responses above background evident only on 6th shock. Temp. 17° C. *A*, recording from the entire animal; *B*, slit recording.

Attainment of plateau level of response probably involves the interaction of two factors, the relative importance of which varies with the physiological state of the specimen. As Bullock notes (1943), in his detailed analysis of facilitated muscular responses of Scyphomedusae, plateau level indicates a state of equilibrium between accumulation and decay of facilitator during the interposition of a series of stimuli. Progress toward equilibrium should be reflected in a decline in the rate of increment of consecutive responses as plateau is approached, and such a decline, is, in fact, observable in records of long seriated responses at low frequencies (< 2 per sec.).

A complicating factor in the luminescent response, which is, perhaps, not evident in muscular responses, is the intervention of fatigue, which can be ascribed to the exhaustion of photogenic substrate. In some animals, which maintain plateau for long periods at low frequencies of stimulation (< 1 per sec.), large stores of intracellular photogenic material seem indicated; in others, when consecutive responses fall off after plateau is reached, it would seem that levels of photogenic substrate are low, and quickly depleted. Rapid depletion of limited substrate would, in itself, hasten attainment of equilibrium, and reduce the apparent magnitude of facilitatory increment. At low frequencies of stimulation, this effect can be avoided by selecting

specimens and records that present maintained plateau-states, but no remedy is available for experiments involving high-frequency stimulation.

In their observations on *Renilla*, Buck & Coyle (1955) have noted peculiar variations in intensity after plateau was reached during protracted stimulation. Occasionally a flash is dropped out, at other times an intercalated flash appears of heightened intensity; sometimes a series appears of depressed intensity, and this in turn is succeeded by several waves which gradually attain plateau level once more. Although I have not attempted to repeat these observations at length, I can confirm these peculiarities. Since these workers were unable to obtain quantitative data on intensities, I am supplementing their observations by recordings (Fig. 3, *E*, *F*), which illustrate the variation in intensity of consecutive responses sometimes encountered. The brightness which attends an intercalated wave can be ascribed to heightened facilitation engendered by the short interval between successive nervous impulses (Fig. 3, *F*). Repetitive firing is here indicated. But variation in intensity of regularly spaced waves, after plateau is reached, appears to reside in some fluctuation in excitability of the effector.

Raising the frequency of stimulation (up to 3 per sec.), the number of shocks being kept constant, brings about an increase in light intensity. That is to say, the light intensity with a burst of 10 shocks at 2 per sec. is greater than with an equivalent burst at 1 per sec.; and, similarly, the light intensity with a burst at 3 per sec. is greater than at 2 per sec. (Fig. 3, *A–C*). At frequencies above 3 per sec., however, an inverse relationship becomes operative, and response-intensities decline (Fig. 3, *C*, *D*). Factors regulating intensity of response are discussed more fully in later sections.

The above responses were judged characteristic of the majority of animals examined, except for several lots of specimens collected during the winter months (January and February). These latter specimens proved to be excessively irritable and excitable; their responses were unpredictable, and unsatisfactory for controlled quantitative studies. Some of the peculiarities evinced by these specimens were as follows: they frequently flashed on the first (electrical) stimulus; one or a few shocks gave rise to a bright durable glow which affected the whole surface and seemed to scintillate rapidly; responses succeeding the initial bright glow were weak in intensity. These animals also seemed to require much less dark-exposure before becoming luminescent: some animals gave bright localized glows under tactile stimulation immediately on transferring them from sunlight to darkness, and others gave propagated luminescent waves (cf. p. 310). No satisfactory explanation for these variations in excitability was found.

EFFECTS OF VARYING STIMULATING VOLTAGE

Previous studies on the nerve net of coelenterates have established that conduction is non-decremental and that, above threshold voltage, further increment in intensity of stimulus does not increase the strength of the muscular response (sea anemones, medusae). The nerve net thus exhibits normal properties of discrete nerve fibres

(Pantin, 1935*a*; Bullock, 1943). In view of these results it was not anticipated that varying the strength of stimulus would alter the response in *Renilla*. However, some experiments of this kind were carried out to determine, for empirical purposes, the threshold necessary for stimulation. Above threshold the luminescent response of *Renilla* is unaffected by stimulus strength over a range of 5 to 30 times threshold intensity (Fig. 5). These effects were to be expected. Indeed, Buck & Coyle (1955), in a forthcoming paper, note that when the stimulus-strength is increased as much as 20-fold during plateau of a series of responses, no increase in intensity of luminescence is observed (visual observations). However, with very strong stimuli (5–30 times threshold, i.e. peak condenser voltages of 45–240 V.), I found that a peculiar effect appeared: the animal responded to the first shock with a very bright flash. The simplest explanation would be to consider this the result of

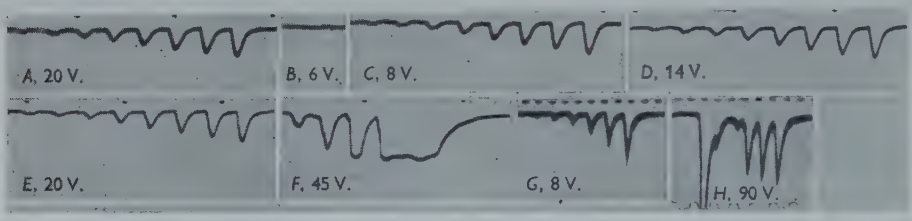

Fig. 5. Effect of different voltages on the luminescent response. *A–F*, one specimen; *G–H*, another specimen. The first specimen (*A–F*) was stimulated with a burst of 10 shocks; the first response appeared on the 2nd shock. Voltages shown are charging voltages on the condenser; stimulating voltages on the animal would be less than these. Recordings from the whole animal; stimuli are not shown in these records. Threshold voltage was 8 V., above which no further increment in response intensity was recorded, but at the highest voltage explored (45 V.), the animal gave a flash to the 1st shock and responded to the next 4 shocks by a series of prolonged glows. Records *G–H* are from another animal in which threshold was 8 V., and which responded to maximal voltage of 90 V. by a very bright flash on the 1st shock, and 1 flash per stimulus thereafter (bursts of 7 shocks used in this specimen). Time scale *A–F*, 14 per min.; *G–H*, 72 per min. Temp., 1st specimen, 17° C.; 2nd specimen, 20·1° C.

direct stimulation of the luminescent cells (photocytes). This is obviously not so since there is no progressive increase in height of response to the first shock when the voltage is raised; rather the bright flash on the first shock appears suddenly at some high voltage and invades the whole rachis. The effect stands in contrast to the orderly, facilitated and predictable responses which can be evoked by repetitive stimulation near threshold strength. It may be noted, however, that *Renilla* often shows supernumerary flashes, intercalated between regularly induced responses; such supernumerary flashes are indicative of repetitive discharge (Fig. 3, *F*). Pantin (1935*c*, *d*) has noted such repetitive firing in muscular responses of anemones, and states that after the intensity is raised to about 10 times threshold (*Calliactis*), the primary response may be followed by one or more extra contractions. A possible explanation of the initial bright flash in *Renilla* is that a very strong shock causes repetitive firing in the nerve net, i.e. sets off several impulses at very short intervals, and these are responsible for a facilitated bright flash.

TEMPORAL CHARACTERISTICS OF THE LUMINESCENT RESPONSE

The transmission speed of the luminescent wave has been determined by Parker (1920*b*) and Buck (1954) by a visual method. There is a fair amount of individual variation. Buck finds a conduction rate of 4·5 cm./sec. at 21° C.; Parker, an average rate of 7·7 cm./sec. at 21° C., and 6·5 cm./sec. at 15° C. According to the latter worker, the Q_{10} for conduction speed is approximately 2. In recordings from the entire animal the latent period between stimulus and beginning of response is 0·5 sec., and maximal height is reached in 0·9 sec. after the first deflexion (at 18° C.). The total duration of the luminescent wave, from first deflexion to extinction, is 4·5 sec.; this includes a long tapering decay period. Half decay from maximal deflexion occurs in 0·4 sec. At least 1 sec. of total response time is occupied by transmission of the luminescent wave across the animal (7 cm. in diameter); most of the long tapering decay period of the curve, occupying 2·5 sec., is due to the slow durable glow of the autozooids. This allows about 1 sec. for duration of a wave across 1 cm. of wave front, and the total duration of the luminescent flash at any one locus is of the order of 0·9 sec. or less.

EFFECT OF HIGH FREQUENCY STIMULATION

When specimens are stimulated with condenser shocks at increasing frequencies, it is observed that the intensity of responses rises with shortening of stimulation-interval, up to some peak value, beyond which further increment of frequency actually brings about a decline in maximal intensity of response (Fig. 3, *C*, *D*). In the graphs of Fig. 6 I have plotted the results of an experiment in which the frequency was increased stepwise from 1 per sec. to 7 per sec. (curve I), and subsequently decreased over the same range (curve II). The intensity of response augments greatly at intervals less than 1 sec., reaches a maximum around 0·5–0·33 sec., and falls off sharply at shorter intervals.

These records are obtained from the whole animal, and their interpretation is complicated by a combination of factors. The rate of transmission of the luminous wave is 5–7 cm./sec. In an animal which is stimulated at one margin, and in which the disk diameter is 7 cm., as in the present case, there will be only one luminescent wave on the rachis at any one time at frequencies of 1 per sec. or less. At low frequencies the records, consequently, show discrete deflexions, each one falling to base-line. At higher frequencies the deflexions begin to fuse, and fusion appears complete at 4 per sec. or more (Fig. 1, *E*). This results from the summed light intensities of several waves proceeding over the surface of the rachis at one time, and from summation of the prolonged glow of autozooids. Recordings from the whole animal, therefore, will give only limited information about the temporal characteristics of the response curve at any one locus.

A large part of the increment of light intensity which attends increased frequency of stimulation is due to instrumental integration of separate waves running simultaneously over the rachis; part is owing to summation of responses of autozooids; some proportion is ascribable to facilitation. At high frequencies, above 3 per sec.,

as already noted, the animal fails to respond to each stimulus, either initially, or after stimulation has been in progress for a short time, and flashes at a slower rate. Decline of ability to respond 1 : 1 to high-frequency stimulation (3 per sec.) probably can be ascribed to lengthening of absolute or relative refractory period, or both. This places a limit on the maximal intensity which can be attained by raising the frequency to a peak at about 3 per sec. In experiments involving muscular responses of sea anemones (*Calliactis parasitica*), Pantin (1935*a*) has found that the threshold rises when a series of stimuli is applied. Although this effect is indicative of lengthening of absolute refractory period, intervention of local insensitivity under

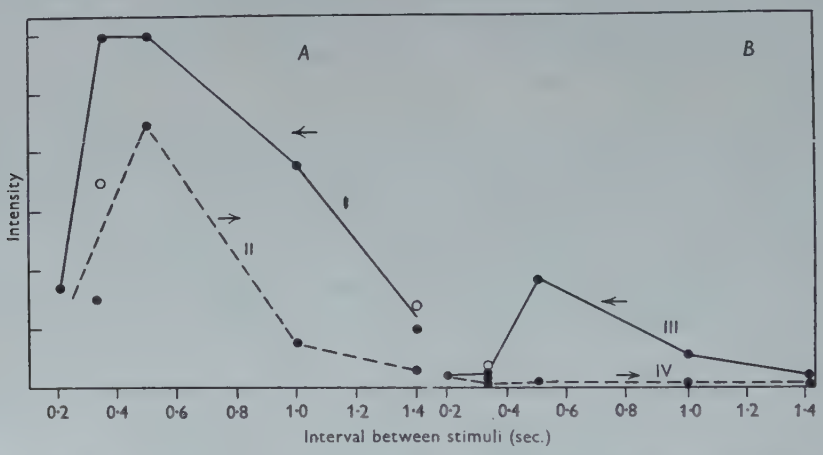

Fig. 6. Investigation of fatigue by the use of two pairs of electrodes. Stimulation consisted of bursts of 10 shocks at frequencies varying from 42 to 240 per min. Each point represents the response to the 10th shock. *A*, first run with one pair of electrodes, in the direction of increasing frequency (solid line, curve I), and repeated in the direction of decreasing frequency (broken line, curve II). *B*, second run with another pair of electrodes lying on the opposite side of the animal. Again, this consisted of a stimulation-sequence in the direction of increasing frequency (solid line, curve III), followed by a run with decreasing frequency (broken line, curve IV). Frequencies and temporal sequence of stimulation were identical in series *A* and *B*. Intensities (ordinates) in arbitrary units. ●, 1st determination; ○, 2nd determination at one frequency. Temp. 16° C.

repeated stimulation may also be a contributory factor. The fall-off in intensity at higher frequencies is occasioned by some form of exhaustion or fatigue. This is emphasized by the reduced responses which persist at a low level after a period of high-frequency stimulation (cf. curves I and II in Fig. 6).

FATIGUE OF THE LUMINESCENT RESPONSE

It has just been noted that high-frequency stimulation produces some kind of fatigue such that subsequent responses are lower in intensity. Increase in refractory period of the conduction system is one effect of high-frequency stimulation (see preceding section). Another factor occasioning decline in consecutive responses under repetitive stimulation is progressive exhaustion of photogeny. This effect has been investigated in several ways. Animals have been subjected to consecutive bursts of

stimuli, and amplitudes of responses have been determined from photographic records. With rapid rates of stimulation (bursts of 10 shocks at 3 per sec.), there is a rapid fall-off in intensity of consecutive response-sequences. The course of decline of intensity is much less at slower rates of stimulation, and some specimens often maintain response-intensity with but slight diminution through many consecutive bursts.

In order to determine whether the decline in intensity of response at high frequencies, as shown in Fig. 6, *A*, is a peculiarity of high-frequency stimulation (fatigue in the transmission system), or exhaustion of photogeny, the following experiment was devised. An animal was stimulated at a slow rate for a given

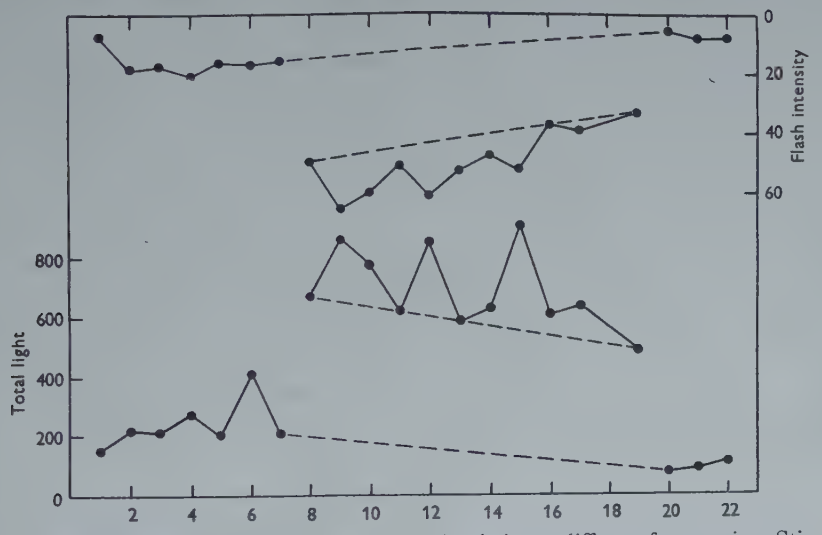

Fig. 7. Fatigue of photogeny induced by repetitive stimulation at different frequencies. Stimulation consisted of 7 bursts of 10 shocks at 1 per sec., followed by 12 bursts of 5 shocks at 3 per sec., and terminated by 3 bursts of 10 shocks at 1 per sec. Ordinates: left, total light produced; right, maximal intensity of flash to last pulse in each burst (5th or 10th). Abscissae, consecutive responses. Temp. 16·2° C.

number of pulses; after several bursts at this frequency, stimulation was continued with fewer pulses at a higher rate, such that the amount of light emitted was about the same as that produced previously. After continued stimulation for some time, the stimulation frequency was switched again to the initial rate. In practice this experiment was difficult to carry out exactly as planned, since it was not possible to gauge the total light produced until the experiment was completed and the records examined. A compromise was achieved by delivering 10 shocks at a slow frequency and 5 at a higher frequency. Under these conditions it was found that the temporal decay-curves for maximal intensity and total light emitted at low and high frequencies ran a parallel course (Fig. 7). The rate of decrease of light intensity occasioned by high-frequency stimulation about matches the reduction measured under low-frequency stimulation, and the decline would appear to be occasioned by exhaustion of photogeny.

The possibility of local fatigue or accommodation in the nerve net in these experiments has been excluded by stimulating alternately with two pairs of electrodes. Each pair rested on opposite extremities of the rachis. The animal was stimulated, via one pair of electrodes, with bursts of shocks, stepwise, with increase in frequency: then with a reverse series, with decrease in frequency. After this run the stimulation series was repeated with the other pair of electrodes. Results of an experiment are presented graphically in Fig. 6. At high frequencies (2–4 per sec.) fatigue intervenes of the type described in previous paragraphs (Fig. 6, *A*). On shifting the locus of stimulation to the second pair of electrodes, the responses elicited remain below those of the first series in intensity, and the response curve, although parallel to the first, remains at a lower level throughout (Fig. 6, *B*). It is concluded that fatigue, as here engendered, is widespread, involving the whole rachis.

After being subjected to strong mechanical stimulation, some animals showed reduced responses for some time subsequently. This effect was only observed after repeatedly administering strong tactile stimuli, which culminated in persistent afterglow and rapid scintillations playing over the surface of the rachis for some 30 min. or more (frenzy-effect of Buck). After the disappearance of this post-stimulatory light, the responses induced by electrical stimulation were very weak at first, and sometimes lacking. With continued trials of electrical stimulation, responsiveness, as measured by intensity of flashes, gradually returned. The course of recovery occupied 10–60 min., probably varying with the individual and the intensity of previous mechanical stimulation. Recovery curves show a definite trend towards increase in intensity but are somewhat irregular, probably owing to the necessity of readjusting electrodes during subsequent expansion of the rachis, thereby introducing adventitious excitation. Strong mechanical stimulation of the kind here employed, also produces very strong contraction of the whole rachis, and subsequent expansion takes place slowly over the course of the next hour. The relation, if any, between state of contraction and relaxation of the body, surface area and luminescence, is not known.

RECOVERY OF LUMINESCENCE DURING DARK EXPOSURE

It has been clearly established that luminescence in *Renilla* is inhibited by exposure to light. When tested by mechanical (tactile stimulation), a previously illuminated animal shows occasional localized points of light in the area of stimulation within 25 min. of being placed in the dark. After 45 min. in the dark, localized responses and luminescent waves are elicited, and the response apparently reaches maximum in 1 hr. (Parker, 1920*b*). According to Buck (1953), inhibition by illumination takes place in the photogenic tissue, and not in the sensori-neural system.

The time course of recovery of luminescence in the whole animal has been followed by placing specimens in the dark, and recording the luminescent response, induced by electrical stimulation, at frequent intervals. Animals were stimulated electrically with bursts of shocks (1–3 per sec.) at 10–30 min. intervals.

Data from some experiments showing increase in intensity of response with time

of dark exposure are plotted in Fig. 8. There is a good deal of variation among different animals. Curve *A* represents recovery in specimens stimulated at intervals with bursts at 2–3 per sec. The response increases slowly during the first half-hour in the dark and levels off after about 70 min. Curve *B* presents the results of a similar experiment, except that a frequency of 1 per sec. was used. With some other specimens the response curve reached maximal plateau value after 2 hr., and some animals showed subsequent decline in response-intensities owing to fatigue. The data are important in emphasizing that an animal should be placed in the dark for about 2 hr. before experimentation begins, in order that comparable quantitative results, from measurement of light intensities, can be secured uncomplicated by recovery of the photogenic reaction in darkness.

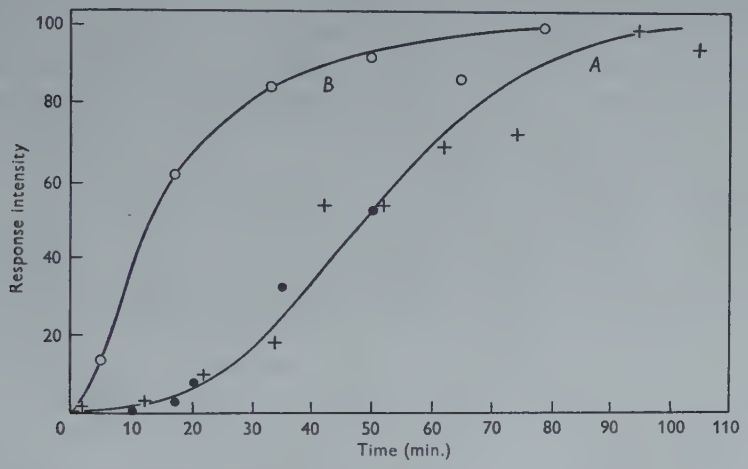

Fig. 8. Recovery of luminescence in animals kept in the dark after previous exposure to daylight. Reappearance and increase of luminescence with time were followed by stimulation with bursts of shocks at intervals of 10–30 min. Curve *A*, results from 2 specimens stimulated with bursts of 5 shocks at 3 per sec. (●) or 10 shocks at 2 per sec. (+). *B*, results from a specimen stimulated with bursts of 10 shocks at 1 per sec. Abscissa, time in minutes after placing animal in dark. Temp. of experiments, 15·3–18° C.

All the recovery curves obtained possess a distinct sigmoid shape. This may represent the true course of recovery of photogeny, but the possibility also exists that the process is complicated by the synchronous progress of fatigue. It is, of course, impossible to determine time-course of recovery in the intact animal without recourse to stimulation and the possibility of causing fatigue. With continued stimulation, progressive consumption of photogenic material would tend to lower the recovery curve without altering greatly its shape.

The level at which inhibition of photogeny by light takes place in *Renilla* is unknown. In ctenophores there is evidence that light acts directly on photogenic material, causing inhibition of luminescence (Harvey, 1952). In some hyper-excitable winter specimens, mentioned previously, it was found that even direct exposure to strong sunlight failed to prevent the appearance of luminescent waves. Buck (1954) mentions that sea pansies exposed for 5–30 min. to bright sunlight,

showed low threshold and bright luminescence when stimulated immediately after bringing them into the dark. These observations would suggest that light is affecting the excitatory mechanism. In those specimens of *Renilla* in which the luminescent response is inhibited by light, it can be demonstrated, by grinding them up in a mortar in the dark, that much luminescent material is still present in the animals. Such light-exposed specimens give off a brilliant glow when so treated. It follows, then, that the photogenic material is not subject directly to inhibition by light, which must, therefore, be affecting some part of the excitatory mechanism.

Light-inhibition can be shown to be quite local by shielding various parts of the animal and then exposing it briefly to illumination. Under these conditions it is discovered that only the exposed region is inhibited, and that concealed regions retain their luminescent ability, i.e. the effect of light is localized to the region illuminated. To test the possibility that light might be acting on the nerve net and thus blocking transmission, the following experiment was carried out. Either whole animals or strips of animals, which had been held in darkness, were covered over with opaque material except for a narrow aperture which completely exposed a narrow band across the whole surface of the disk or strip. The preparations were then exposed to the light of a tungsten lamp of intensity sufficient to inhibit luminescence (100 W. at 30 cm.). After extinguishing the light and removing the opaque shield, the preparation was then stimulated electrically. It was found that in such preparations, with the electrodes resting at one extremity, the luminescent wave arose under the electrodes, progressed up to the previously illuminated area, which now appeared dark, and, after a brief interval, reappeared on the other side of the animal. It is concluded from these experiments that illuminating a strip of rachis, so as to expose a band across the entire nerve net to light, does not abolish the transmission of excitation concerned with luminescence.

Inhibition by light, therefore, must occur terminally, and affect the entire photogenic surface. Its locus of action involves either the neuro-effector junction or the photocyte itself, apparently proximal to the photogenic material.

FACILITATION

The existence of facilitation in the luminescent response is revealed by two kinds of evidence. First, one shock is ineffective, and two or more shocks are required to elicit a response. Secondly, at slow rates of stimulation, producing discrete flashes, consecutive flashes increase in intensity, and the amount of increment is proportional to the rate of stimulation, within certain limits.

Facilitation is considered by Buck (1954), who presents various reasons for believing that it occurs peripherally at the photocytes, and not in the interneural synapses of the nerve net. The most cogent of the several arguments advanced is that transmission never appears decremental, i.e. a luminescent flash, once evoked, sweeps across the whole surface of the rachis without apparent fading. One instance of interneural facilitation may be recalled from Pantin's studies (1935*a*, *b*) on *Calliactis* in which he found that a few impulses produced a localized response of

the disk, and that the response could be caused to radiate over more and more tissue by increasing the number of shocks.

The possible existence of interneural facilitation was attacked experimentally by investigating the effect of reduction of area of neural pathways on onset of flashing and transmission of excitation. The arguments are these: if facilitation is occurring throughout the nerve net, then reduction of available pathways may well result in more impulses being required to overcome synaptic resistance in the limited avenues still available. Or conversely, if repetitive stimulation induces synaptic fatigue, then paring down the transmission route may produce a condition in which interneural facilitation can be revealed during prolonged stimulation. The experiments were carried out as follows. Animals were anaesthetized with $MgCl_2$, and an incision was made part way across the rachis; the parts of the rachis were left connected by a narrow bridge about 2 mm. in diameter. This was about the smallest bridge which could be left, without completely blocking conduction by trauma. Other animals were partially transected twice so as to leave narrow alternate bridges on opposite sides of the rachis (Fig. 9). Subsequently, the specimens were placed in sea water for several hours.

These specimens were stimulated electrically at frequencies ranging from 1 per 2 sec. to 3 per sec. Fifteen animals were studied. Observations were made at room temperature (25–26° C.). The effects which were observed varied from specimen to specimen, but the following is an indication of the kinds of luminescent responses secured (Fig. 10).

Several shocks were necessary to bring up the first response in the tissue proximal to the first cut, as in intact animals. In some animals the first luminous wave induced successfully crossed the bridge to the region of the rachis distal to the cut and, when two cuts had been made, continued across the second bridge to the third rachidial region. With continued stimulation, subsequent waves were transmitted to other regions of the rachis in the same manner (Fig. 10, *A*). With other specimens, the first wave, or first few luminous waves, were confined to the region proximal to the cut, and the second or some subsequent wave crossed the first and second bridges (Fig. 10, *B*, *C*, *D*). These relations probably depend on differences in extent and degree of injury to the residual bridges of tissue. There were definite indications that, initially, continued stimulation favoured the transmission of excitation across one or more bridges. Transmission from proximal to middle and distal regions continued regularly for long periods in some specimens at slow rates of stimulation (intervals of 1 sec. or longer). In other animals, transmission soon became irregular so that there were often long periods in which no waves crossed over medially and distally, or crossed to the medial segment only, followed by periods in which many waves crossed to the medial and sometimes to the distal regions.

With long continued stimulation in some specimens, there was a tendency for transmission across the bridges to drop out to a greater extent, or even to cease completely. This effect was more pronounced at higher frequencies (above 1 per sec.), but was difficult to record vocally on the tape recorder because of the speed at

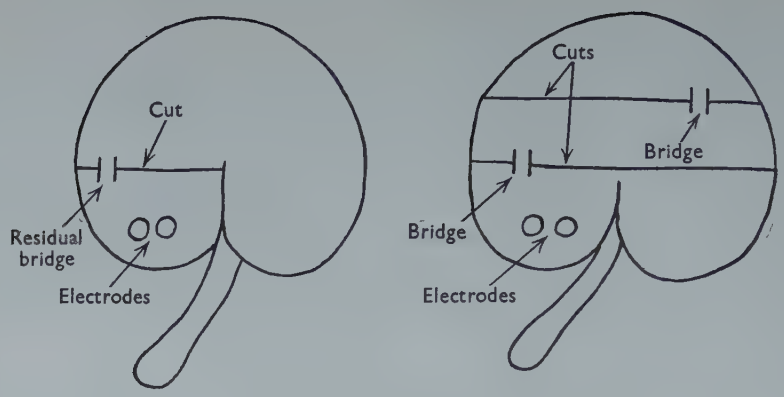

Fig. 9. Diagrammatic representation of pattern of incisions made in rachis of *Renilla* to investigate facilitation (explanation in text).

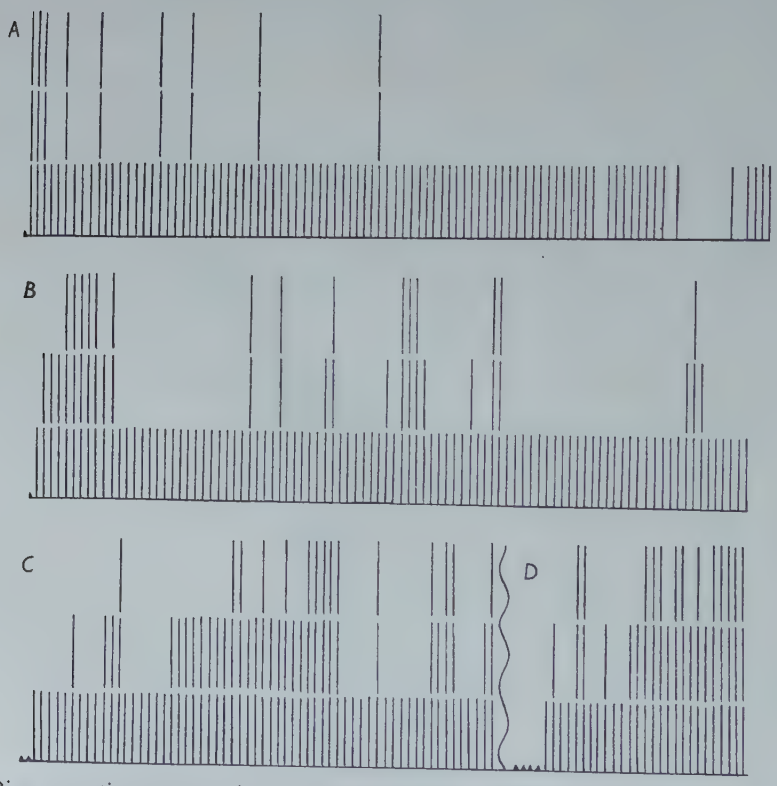

Fig. 10. Diagrammatic representations of the responses of partially transected animals to repetitive stimulation. Two cuts were made across the animal at different levels, so as to produce three rachidial regions connected by two narrow bridges. In each diagram, the vertical lines represent luminescent responses to stimuli: bottom row, responses of proximal region under electrodes; middle row, responses of central piece; upper row, responses of third, distal region furthest from electrodes. Small pips, bottom left, are initial stimuli before onset of responses. *A*, *B*, *D*, stimuli at 1 per sec.; *C*, stimuli at 42 per min. Experiments carried out at room temperature, 25–26° C.

which the complex succession of waves was taking place. There were also instances in which, after long continued stimulation, the successive waves in the proximal region (around the electrodes) fluctuated greatly in intensity, waning and waxing in brightness, and were even interrupted by dark intervals in which one or several waves dropped out of the series. Buck & Coyle (1955) have described the same effect in whole animals. But more interesting, it was observed that when waves were only occasionally crossing to the middle and distal fragments, it was usually, but not invariably, the bright waves which crossed over. This raises the question, which cannot yet be answered, as to whether the fluctuations in intensity of response, as here observed, are owing to changes in the nerve net, rather than to changes at the neuro-effector junction. Finally, the responses of the middle and distal regions were usually much dimmer than those of the proximal region, when only occasional waves were crossing over; when fewer waves were crossing over to the distal region than reached the middle region, the responses of the former were weaker than those of the middle region. These differences in the brightness of responses in the three regions were probably the result of different levels of facilitation. If fewer impulses are reaching, say, the middle than the proximal region, the time interval between arrival of excitation at the photocytes in the middle region will be greater, decay of facilitation will have progressed further, and responses will be correspondingly weaker, than in the proximal region. These experiments provide evidence for interneural facilitation, and are discussed in the next section.

DISCUSSION

Luminescence in *Renilla* takes the form of discrete flashes of light which course over the surface of the rachis. Once a facilitated path is established through to the photocytes, there is a wave of light corresponding to each electrical stimulus, and it is highly probable that each wave corresponds to the passage of a nervous impulse across the nerve net. Evidence has been secured that the nerve net is unpolarized and transmits a wave of excitation equally well in all directions. The rate of passage of the luminous wave is an index of the speed of transmission of the underlying nervous impulse. The nervous impulse is exciting successive trains of zooids, and the width of the wave front is obviously determined by the duration of the response in each zooid.

Renilla is a colonial animal, and its nerve net not only serves the individual zooids, but provides a system of communication among the constituent zooids of the colony. The majority of studies on coelenterates, heretofore, have been conducted on separate individuals, particularly medusae and actinians. The luminescent responses of *Renilla*, on the contrary, provide a very sensitive index of transmission in the nerve net of a compound form, and reveal that transmission in such systems is essentially like that in non-colonial coelenterates.

The luminescent response of *Renilla* conform to the neuro-effector pattern established for other coelenterates (medusae, actinians), viz. that facilitation is operative in establishing the overt response. Several impulses are necessary to

produce the first response and consecutive responses increase in magnitude, contingent upon accumulation of facilitator. This is essentially like the condition seen in the marginal sphincter of *Calliactis*, except that the briefer duration of the luminescent response allows separate flashes to be fully resolved, each immediately succeeding its effective stimulus (Pantin, 1935*a*). Summation is nevertheless possible at frequencies above 1 per sec., and this summation probably occurs both in the short flashes of the siphonozooids and the longer glowing of the autozooids, the relative contribution of each depending on the conditions of stimulation. This is in contrast to conditions in medusae where absolute refractoriness (*c.* 0·7 sec.) prevents fusion of consecutive contractions (Bullock, 1943). Facilitation occurs terminally, at the junction between nerve fibres and the photocytes, or in the latter.

Facilitation of luminescent responses is now known to occur in several animals. Harvey (1917), studying another pennatulid *Cavernularia*, found that one shock was usually ineffective, and three or more pulses were required to bring out a response. I have found that facilitation is operative in the luminescent response of *Beroe* and polynoids, in which a flash is produced by the first shock, but subsequent flashes increase in amplitude (Nicol, 1953, 1954, 1955). Neuro-effector facilitation is thus one factor regulating the luminescent response. In *Renilla* its operation demands that several impulses impinge upon the photocytes before they respond, while continued stimulation effects a stronger response.

Under normal conditions a luminescent wave, once established, invades the entire rachis, and is evidence that each nervous impulse is effective in traversing the whole nerve net. Nevertheless, Buck (1954) has noted a few instances (about six in several thousand observations) of failure of a wave to affect the whole rachis. One specimen, which had been exposed to sunlight, clearly showed decremental conduction: the response, at first local, increased in brightness and spread out further with each succeeding shock until the whole rachis was involved. The present studies have demonstrated that decremental conduction can be made evident by partial transection, with the result that several additional impulses are necessary to further transmission across the narrow bridges so produced, or continued stimulation can introduce blockage across such avenues. It would appear that normally transmission is always effective between the elements of the nerve net, but that breakdown occurs under certain abnormal conditions such that several impulses are required to establish through-conduction between separate parts of the nerve net. Under such conditions interneural facilitation becomes operative in through-transmission.

The failure to transmit impulses which develops in partially transected animals during prolonged stimulation is evidence of fatigue in the nerve net, presumably at synaptic boundaries. Histological and functional studies have not yet been carried out to determine the configuration and anatomical locus of the net in *Renilla*, e.g. length, position and course of conduction axons, occurrence of synapses, and presence of alternative pathways at superficial and deeper levels. Three facts, however, suggest the existence of a meshwork of many small neurones, rather than long conducting pathways, viz. equal ability to transmit in all directions, widespread, closely spaced and diffuse distribution of photocyte effectors, and introduction of

interneural facilitation and fatigue by transection experiments. It would appear as if the whole surface of the rachis were provided with a meshwork of short distance neurones, at least so far as the luminescent response is concerned. It may be recalled that Bullock (1943) found that through-transmission was the rule in the nerve net of Scyphomedusae, but evidence for interneural facilitation between elements of the nerve net could be secured by depression with magnesium excess, in which event continued stimulation caused the muscular response to invade increasingly greater areas of the bell. It is as if the elements of the nerve nets of *Renilla* and Scyphomedusae were geared to 1:1 transmission, but this broke down under fatigue or magnesium anaesthetization, so that repeated stimulation was required to overcome heightened resistance to synaptic transmission.

Renilla is one of the several animals in which it has been established that illumination produces inhibition of the luminescent response. Other known instances are certain flagellates, viz. *Gonyaulax* (personal observation of F. Haxo), and *Noctiluca* (contradictory observations, see Harvey, 1952); coelenterates, viz. sea pens *Pteroeides*; and ctenophores in general (segmenting eggs and adults) (Harvey, 1952).

Light inhibition in *Renilla*, and perhaps in other coelenterates, occurs terminally in the region of the photocytes. In ctenophores it has been established that light affects the photogenic material directly, but this is not the case in *Renilla*, where it acts on some terminal mechanism, in or near the photocyte. Conditions in dinoflagellates are still largely unexplored. It is probably significant that nearly all these instances concern animals that either lack a nervous system, or possess a nerve net, in contrast to animals which possess a central nervous system not normally subject to direct light inhibition. In *Renilla* the unpolarized nerve net regulates several responses, including withdrawal of the polyps, contraction of the rachis and expulsion of water, and luminescence. All these activities are evoked by tactile stimulation, although it is possible that they are subject to different degrees of facilitation. It has been shown that tactile stimulation, besides causing contraction and an immediate luminescent response, also alters the excitability of the nerve net so that subsequent luminescent responses are affected. We may concede that probably the same net and efferent pathways are involved in exciting luminescence as in producing the muscular responses just mentioned. Peripheral inhibition of photocytes or photogenic material by light, therefore, is a mechanism which blocks luminescence in daylight, even in the presence of neural excitation, and conserves photogenic material for periods of darkness, when the luminescent response might be of use to the animal.

A third factor affecting the luminescent response is variable excitability of the nerve net and repetitive discharge. A strong tactile stimulus usually gives rise to a train of waves, and prolonged electrical stimulation is often succeeded by long after-discharge. After-discharge and maintained rhythmic flashing are properties of the nerve net. Under moderate conditions of excitation, the stimulated area continues to act as pacemaker after stimulation has ceased; the heightened excitatory state there engendered gives rise to rhythmic discharge in the nerve net, resulting in luminescent waves. As Buck (1954) observes, prolonged electrical stimulation some-

times produces an extraordinary hyper-excitable state in which complex waves run all over the rachis. In this condition the entire nerve net is in a state of gross excitation and displays numerous focal points from which waves radiate outwards for short distances. Frequently, such a condition passes through a phase in which the multiple foci diminish in number and are ultimately succeeded by one locus of origin. This presumably represents a stage in which one pacemaker with leading rhythm has taken precedence over the others, and continues in this role until total decay ensues. Rhythmic after-discharge outlasting the stimulus has been recorded in two other luminescent animals (*Beroe* and polynoid worms) (Nicol, 1953, 1954, 1955). In all cases it is due to continued discharge in the nerve net (*Renilla*, *Beroe*) or peripheral ganglia (polynoids). It represents a mechanism by which effective stimulation can bring about a protracted response, which is graded in total duration by number and frequency of flashes. On this background of flashing, summation of the slower responses of the autozooids takes place in *Renilla*, so that the total response becomes one of a shifting persistent glow with superposed luminescent waves. Brief flashes, compared with the alternative possibility of a persistent glow, variable in duration, may well possess ethological significance in terms of visual attention, but this is entirely speculative.

SUMMARY

1. Luminescent responses in the sea pansy *Renilla köllikeri* take the form of light waves which run over the entire surface of the rachis. As other workers have shown, the responses are controlled by an unpolarized nerve net, and are subject to facilitation. Records obtained by photoelectric techniques are furnished to show certain features of the response.

2. At moderate rates of electrical stimulation (1 per sec.), 2–3 shocks are required to evoke a response. At slower rates, more stimuli are necessary owing to decay of facilitator. At frequencies above 3 per sec., the response rate is slower than the stimulation frequency, owing to refractoriness in the conducting mechanism (*c.* 0·2 sec.).

3. Summation occurs at frequencies above 1 per sec. This results largely from fusion of the more persistent glowing of autozooids. Siphonozooids are responsible for the brief flashes.

4. Maximal estimates of latent period and flash-duration are of the order of 0·5 and 0·9 sec. respectively.

5. With repeated stimulation, the response intensity is subject to decay. Experiments suggest that this is a consequence of exhaustion of photogenic material.

6. On mechanical stimulation, luminescent waves are evoked at first; but with continued stimulation, refractoriness sets in, and responses become localized to the area of stimulation. Subsequent electrical stimulation reveals that the transmitter mechanism is fatigued, and excitability gradually returns over the course of the next 30–60 min.

7. Luminescence in *Renilla* is inhibited by illumination. When an animal is placed in the dark, luminescence gradually returns over the course of the next 1–2 hr. A form of recovery curve, derived from consecutive periods of electrical stimulation is presented. This has a sigmoid shape. Inhibition of luminescence occurs terminally, in or near the effectors (photocytes).

8. Facilitation of luminescence, as measured in these experiments, occurs terminally, at the neuro-effector junction or in the photocytes, and not interneurally, in the nerve net.

9. By deep incisions, which almost completely transect the animal except for narrow residual bridges of tissue, it has been possible to produce experimental conditions in which several conditioning impulses are required to bridge the tissue junction. Continued stimulation of such preparations produces fatigue of the transmitting mechanism at such loci. These experiments provide evidence for interneural facilitation and fatigue in the nerve net under particular conditions. Under normal circumstances, however, interneural transmission is 1:1 throughout the nerve net.

10. The results obtained on *Renilla* are compared with findings on other coelenterates.

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A SIMPLE PROCEDURE FOR THE STUDY OF IONIC REGULATION IN SMALL ANIMALS

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INTRODUCTION

In the study of ionic regulation in small animals, such as insects or small crustaceans, the quantities of blood or excretory fluid available for analysis are often not greater than about 1 ml. This volume might contain anything between 0·1 and 50 μg. of any one of the inorganic ions. The larger amounts can be estimated by the conventional micro-analytical methods, but measurements around the 1 μg. level require special ultra-micro-techniques.

The methods which have already been described for the determination of inorganic ions in quantities of 1 μg. or less are of two kinds: first, there are the ultra-micro-chemical methods which are generally adaptations to capillary technique of conventional chemical methods of analysis; and secondly, there are methods which rely on the exploitation of some particular physical property of these substances. Among these latter methods may be mentioned the technique devised by Engstrom (1946) for the quantitative estimation of a number of elements by X-ray absorption spectrography, making use of the absorption discontinuities which appear at a characteristic wavelength for every element. Most of the biologically occurring elements can be estimated in this way, and the quantities which can be measured are extremely small (10^{-3}–10^{-6} μg.). More recently, the technique of flame photometry has been developed on a micro-scale (Ramsay, Brown & Falloon, 1953) for the determination of quantities of sodium and potassium in the order of 10^{-5} μg.

If very small quantities are to be measured then undoubtedly these physical methods are of the greatest value, but the more conventional chemical methods have the advantage that they provide a simple means of estimating all the inorganic ions and only make use of apparatus which can be easily and cheaply constructed.

A number of ultra-micro-chemical methods (operating at the 1 μg. level) have already been described for some of the important biological ions, and these methods have been reviewed recently by Glick (1949) and by Kirk (1950). In general, the ion is either titrated directly by some specific chemical reaction or precipitated from solution by some specific reagent, and then the precipitate separated from the solution, redissolved and titrated. Table 1 lists a number of these methods and shows the chemical reactions which are used for the estimation.

A wide range of chemical methods have been used which involve many different types of titrations, of which chloride, acidimetric and oxidimetric are examples.

Table 1

Substance	Precipitation agent	Titration	Author
Chloride	Silver nitrate	Excess silver nitrate with sodium thiocyanate	Wigglesworth (1938)
Chloride	—	Direct with silver nitrate using electrical end-point	Linderstrøm-Lang, Palmer & Holter (1935) and Cunningham, Kirk & Brooks (1941*a*)
Sodium	Zinc uranyl acetate	Reduce uranyl ion and titrate with ceric sulphate	Lindner & Kirk (1938)
Potassium	Chloroplatinic acid	Reduced with sodium formate and Cl titrated with $AgNO_3$	Cunningham, Kirk & Brooks (1941*b*)
Potassium	Chloroplatinic acid	Converted to iodoplatinate and titrated with $Na_2S_2O_3$	Norberg (1937)
Calcium	Ammonium oxalate	Excess ceric sulphate and back titrated with ferrous ammonium sulphate	Lindner & Kirk (1937)
Calcium	Ammonium oxalate	Converted to calcium carbonate and titrated with acid	Sobel & Sobel (1939)

Since often the concentrations of a number of ions have to be measured at the same time the work involved in the analysis can be greatly reduced if a standard procedure is adopted for all determinations, where possible the same type of titration being used. This avoids duplication of apparatus or continual cleaning and refilling the micro-burette with different solutions and also means that it is only necessary to become accustomed to the colour change which occurs at one type of end-point. Of the available titration methods, the ultra-micro-methods for chloride are both reliable and easy to perform and this paper describes methods for the estimation of sodium, potassium, calcium and magnesium using this titration in each case. This has the additional advantage that the chloride ion is one of the ions which will probably be measured in any case.

The methods by which the other ions can be converted to chlorides are described below, but first an account is given of the procedure and the apparatus which is common to the methods for all the cations.

COMMON PROCEDURE

The sample (about 1 μl.) is introduced by means of a capillary micro-pipette into the bottom of a small glass tube made from 'Hysil' tubing of 2 mm. internal diameter. The tube is 20 mm. long, is sealed at one end, and at a point 5 mm. from this end the tube tapers down to give a conical base. The contents of the tube are dried at 100° C. and transferred, in a small metal rack, to an incinerating oven maintained at 450° C., and left there until the organic matter has been decomposed. This generally requires from $\frac{1}{2}$ to 1 hr., but the time varies with the amount of organic material present.

When the tubes have cooled, the precipitating agent is added to them so that the ash is covered and each tube is about half full. The mixture is well stirred with a very fine glass rod, the top of the tube is covered with a rubber cap and the tubes allowed to stand until the precipitation is complete. The supernatant is separated by

centrifugation in a 'B.T.L.' semi-micro angle centrifuge, the normal tubes of which have been replaced by lengths of wooden dowel rod into which holes have been drilled axially to take the small tubes. A dozen tubes can easily be accommodated at once in this way. Centrifugation for 2 min. is generally sufficient, and during this time the precipitate is deposited at the bottom of the conical part of the tube and slightly displaced to one side. This displacement, which is caused by the angle of centrifugation, greatly facilitates the removal of the supernatant. This operation is carried out by sucking off the fluid through a micro-pipette with a slightly curved tip, while the tube is inclined at an angle of about 30° to the horizontal with the precipitate on the lower side of the tube. The tube is supported in this position by a small 'Perspex' holder, and the operation is controlled by observation under

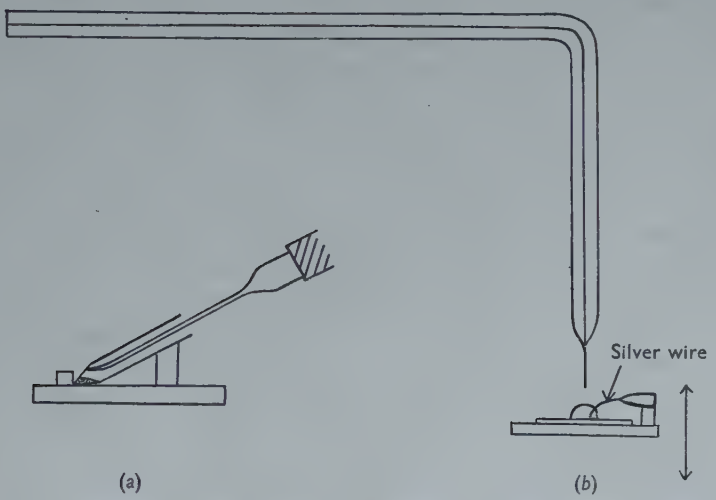

Fig. 1. (*a*) The technique for the removal of the supernatant from the centrifuged precipitate. (*b*) The burette and titration platform with silver electrode in position.

a binocular-dissecting microscope. The relative positions of the tube, precipitate and pipette are shown in Fig. 1. Suction through the pipette is either from the mouth or by connexion to a filter pump, and in this case the suction is controlled by means of a side tube of rubber which can be closed by the fingers.

After the removal of the precipitating agent the precipitate is washed in the appropriate solution twice using the same technique. The separation completed, the precipitate is dried at 100° C. and converted quantitatively to a chloride by one of the methods described below. This reaction is carried out in the same tube and the chloride is washed out from this in a micro-pipette and transferred to the titration platform. Dilute nitric acid is used for washing out when the chloride is titrated by the Volhard method and 0·1 N acetic acid for the electrometric method.

For the titration, the burette and associated apparatus described by Shaw & Beadle (1949) is used since this type of simple automatic micro-burette is very easy to construct and has proved very reliable in operation. The design of the micro-burette has been improved since the original description.

The micro-burette

The micro-burette is made of a length of 'Veridia' thick-walled capillary precision-bore tubing of 0·2 mm. internal diameter, which is bent at right angles and a short tip pulled at the end of the vertical limb (Fig. 1). This gives a much more robust construction than in the previous design. Fluid runs out of the burette when the titration drop on the platform is raised to touch the tip and stops when the drop is lowered if the correct dimensions of the burette are chosen (see below). The titration platform consists of a 1 in. square of glass which has been painted white and then waxed over, or, more simply, a square of polytetrafluorethylene ('Fluon'), the latter having the advantage that it can be cleaned in any organic solvent. The size of the titration drop is usually about 50 μl. and is continually stirred by means of a fine jet of air directed against one side of it.

The correct functioning of the burette depends on the length of the vertical limb; it must be greater than the rise in this limb due to surface tension (h). Now $h=2T/rdg$, where T is the surface tension, d is the density and r, the capillary radius. For water and for a capillary of 0·2 mm. diameter this length = 14·3 cm. On the other hand, the height (h) must not be so great as to cause a drop to be formed at the end of the tip in air. $h_1=2T(r+r_1)\,dg$, where r_1 is the radius of the tip for a hemispherical drop to be formed at the tip. Thus if the tip diameter was the same as the burette diameter then this height would be twice the minimum height. The length of the vertical limb, then, must lie between these two limits and should be nearer the minimum than the maximum for the greatest stability. If the titration drop is small then the minimum height of the vertical limb will be increased by an amount equal to the pressure excess inside the drop $=2T/Rdg$, where R is the drop radius. For a 50 μl. drop this correction would be about 0·7 cm. and would become increasingly important as the drop size was made smaller. The critical dimensions are: (1) the radius of the horizontal limb in which the meniscus lies, and (2) the radius of the tip where the drop forms.

The rate of flow of fluid from the burette must also be controlled so that the meniscus in the horizontal limb of the burette moves at a convenient speed and there is no tendency to over-shoot the end-point. This rate of flow can be predicted by using Poiseuille's equation for the rate of flow of liquid through a capillary. The volume flowing in unit time, $Q=\pi r^4P/8uL$, where P is the pressure, u is the viscosity and L is the length of the capillary. For the burette, P can be measured by the height of the vertical limb less the minimum height.

For a burette of 0·2 mm. internal diameter, of total length 40 cm. and with the vertical limb 21 cm. high, the rate of flow of water from it can be calculated to be $0{\cdot}56\times10^{-4}$ ml./sec. and the meniscus velocity as 1·9 mm./sec. This will be the initial speed when the burette is full; as the burette empties the rate will increase. This meniscus velocity is rather too fast and will be faster still if the capillary diameter of the burette is greater, but the velocity can be reduced by making a fine tip at the end of the vertical limb. The diameter of the capillary must be reduced by a factor of five or ten to bring about an appreciable reduction in velocity but the

longer the tip the greater is the reduction. The most satisfactory way of getting the correct rate of flow is to draw off a long thin tip and then break pieces off until a satisfactory rate is reached. Another way of changing the rate of flow is to alter P, and this can be done by attaching some form of pressure-regulating device to the end of the horizontal arm of the burette. This adds to the complication of the apparatus but does provide a variable rate-of-flow regulator, if this is required.

Chloride titration

The chloride-ion concentration is estimated by either one of two methods. The first of these makes use of the Volhard titration, which was first employed as an ultra-micro method by Wigglesworth (1938), but in this case the burette and associated apparatus described in the preceding section is used. A similar procedure to that described by Wigglesworth for the addition of nitric acid, silver nitrate and indicator is used, and the end-point facilitated by using a white titration platform illuminated with a 'daylight' electric lamp. Using this arrangement and estimating amounts of chloride of about 1 μg., it was quite easy to keep the standard deviation for the estimation of identical quantities down to the $\pm 1\%$ level. The method can, in fact, be used to determine much smaller quantities of chloride than this with little loss of accuracy. By reducing the tip diameter of the burette and the size of the titration drop, and by observing this drop during the titration under a binocular microscope, it is possible to reduce the scale so that quantities as small as 0·01 μg. chloride can be measured to $\pm 2\%$.

The other technique that can be used with the same apparatus is an electrometric method similar to that of Linderstrøm-Lang, Palmer & Holter (1935) in which the chloride is titrated directly with silver nitrate, and the end-point is indicated by the change in potential between two electrodes. The burette is the same as used in the first method except that a piece of platinum wire is fused into the vertical limb of the burette, which together with the silver nitrate in the burette served as one of the electrodes and gave a fixed potential. The other electrode consisted of a fine silver wire which dipped into the titration drop (see Fig. 1). The potential between these two electrodes depends on the concentration of silver ions in the titration drop. This potential was measured with a direct-reading valve voltmeter, and at the start of the titration with 0·1 N silver nitrate in the burette a reading of about 210 mV. is obtained. During the course of the titration this gradually falls to about 170 mV., and then suddenly drops to 70 mV. as the end-point is reached. The titration should be stopped when the potential has fallen to 120 mV. Towards the end of the titration fluid from the burette must be added slowly in order to give time for the new potential to be established and to avoid overshooting the end-point. This is best done by means of a pressure regulator used in the way described above since contact between the titration drop and the burette tip should not be broken during the titration.

The accuracy at the 1 μg. chloride level is $\pm 1\%$, and therefore comparable with the Volhard method. The latter method is the simpler of the two, for the electro-

metric method requires a pressure regulator and a valve voltmeter, but the electrometric method has the advantage that the end-point detection is an automatic one.

Calcium

To the incinerated sample in the precipitation tube is added 50 μl. of a 5% solution of ammonium oxalate adjusted to pH 5 to prevent the co-precipitation of magnesium. The mixture is stirred and allowed to stand for 1 hr. and then the precipitate separated in the manner already described. The precipitate is washed with 50 μl. of concentrated ammonium hydroxide solution which has been diluted 10 times with distilled water. The pecipitate is dried at 100° C. and then heated at 450° C. for half an hour to convert the oxalate to carbonate. The carbonate is dissolved by the addition of 50 μl. of N/5-HCl, and finally heated to dryness at 100° C. The excess HCl evaporates off and calcium chloride is left behind. This is transferred, by washing, to the titration platform and the chloride content measured.

Magnesium

Magnesium is precipitated from solution by the use of 8-hydroxy quinoline. In order to avoid interference from calcium, the precipitation is carried out on the supernatant from the calcium separation described above. The ammonium oxalate supernatant, together with the washing solution, are transferred directly to a precipitation tube. To this tube also is added 1 μl. of a 5% solution of 8-hydroxyquinoline in alcohol and the mixture stirred with a fine glass rod. The tubes are capped, placed in the 100° C. oven for a few minutes to start the precipitation, and then left for half an hour at room temperature for it to be completed. The precipitate is separated in the usual way and washed also with the ammonium hydroxide solution. The presence of the relatively high concentration of ammonium salts does not seem to interfere with precipitation by the hydroxyquinoline, but these salts can be removed, if desired, by evaporating the solution to dryness and heating to 450° C. for a few minutes to decompose them.

The washed precipitate is heated at 450° C. for $\frac{1}{2}$–1 hr. which decomposes the organic compound leaving behind magnesium oxide. This is dissolved in dilute HCl and the solution evaporated to dryness in the same way as in the calcium method. The magnesium chloride which remains is measured as before.

Sodium

Sodium is precipitated by a saturated solution of zinc uranyl acetate as the triple salt, sodium zinc uranyl acetate. The first attempt at converting this acetate to a chloride was by simply heating it with HCl and evaporating the solution to dryness. It was hoped that the acetic acid would distil off followed by the excess HCl and leave behind the chloride. However, trials showed that the yield of chloride was low and variable, possibly due to the formation of basic chlorides. Experimenting with simpler uranyl salts it was found that a much more consistent sample of uranyl

chloride could be prepared by the action of dry HCl gas on the oxide (UO_3). This oxide could not be prepared directly from uranyl acetate by heating as it decomposed at a lower temperature than was required to decompose the acetate, but it was found that the oxide could easily be prepared by heating the acetate below 400° C. with a small quantity of ammonium nitrate. This substance itself decomposes at this temperature and also oxidizes the acetate to leave behind the red oxide.

Tests on a standard solution of uranyl acetate were carried out. The oxide was formed in the usual precipitation tube and converted to chloride by passing dry HCl from an HCl generator into the tube by means of a glass capillary. Formation of the chloride was very rapid and could be seen by a change in colour from red to yellow-green. The tubes then were heated to 100° C. to drive off any HCl and the chloride content measured. The results shown in Table 2 indicate a variation of less than ±2%, but the yield of chloride is only 83% of that expected if the acetate were converted quantitatively to UO_2Cl_2. The experiments were repeated with standard solutions of sodium zinc uranyl acetate and a large number of determinations (see Table 2) again demonstrated that the variation between readings was small (between ± 1 and 2%). The yield for this salt also was between 82 and 83% of that expected, but since this was constant and since the composition of the uranium chlorides is not fully known, the fact that the amount fell short of the theoretical was not very serious. The reaction was used as the basis for the sodium method and a correction applied in the calculation to compensate for the low yield.

Table 2

Substance	Chloride (μg.)	No. of readings	S.D. (μg.)
Uranyl acetate	2·36	10	0·04
Sodium zinc uranyl acetate	3·14	15	0·05
	6·28	14	0·09
	8·23	10	0·09
	9·42	7	0·09

In the complete method 50 μl. of a saturated solution of zinc uranyl acetate is added to the incinerated sample in the precipitation tube and the mixture stirred. The tubes are allowed to stand for half an hour for the completion of the precipitation. The separation is performed in the usual way, and the precipitate washed first with 50 μl. of ethyl alcohol and then with ethyl alcohol which has been saturated with sodium zinc uranyl acetate. The separated precipitate is dried at 100° C. together with a small drop of saturated ammonium nitrate solution. This was heated to 300–400° C. until the oxide was formed and the chloride produced in the manner described above. The sodium content is calculated on the assumption that 7·4 atoms of chloride were equivalent to one of sodium. This figure might be different if the reaction is carried out under different conditions, and should be checked for each new series of sodium measurements.

Potassium

Potassium is precipitated as the chloroplatinate and free chloride ions liberated from this salt by the reduction of the platinum salt with sodium formate (Cunningham, Kirk & Brooks, 1941*b*). A slightly different method was necessary for the preliminary incineration of the sample since contact between the 'Hysil' tube and the sample at high temperatures invariably lead to serious losses of potassium. The sample can be placed on a loop in the middle of a piece of platinum wire which stands upright in the tube so that the sample does not come in contact with the glass. After the incineration is complete, sufficient of a 4% solution of chloroplatinic acid in 80% alcohol is added to the tube to cover the loop in the wire, and the solution is stirred by the wire itself so that the ash comes off the wire and the precipitate is formed in the tube. The wire is removed, and after $\frac{1}{2}$ hr. the precipitate is separated in the usual way and washed with absolute alcohol saturated with potassium chloroplatinate. 50 μl. of 0·2M sodium formate is added to the precipitate, and the mixture evaporated to dryness at 100° C. and the chloride content estimated as before.

RESULTS

Some results obtained by the applications of these methods to the estimation of the four cations, both from solutions of their chlorides and from blood samples, are shown in Table 3. The measurements of potassium, calcium and magnesium all show standard deviations of between $\pm$ 1 and 2% for both the inorganic samples and for the blood samples. Estimations of mixtures of calcium and magnesium indicate that the interference between two is of a low order. With the sodium determinations the error is much greater—the standard deviations are as much as $\pm$ 4%. The larger error in these measurements arises largely during the process of separating the precipitate of sodium zinc uranyl acetate from the supernatant, and this is due to the

Table 3

Substance	Expected (μg.)	Found (μg.)	No. of readings	S.D. (μg.)
Potassium	2·80	2·78	6	0·03
	1·46	1·46	8	0·02
Blood	—	0·84	5	0·01
Calcium	1·60	1·61	8	0·03
	3·20	3·18	6	0·04
	11·50	11·44	5	0·18
+added Mg	11·50	11·53	9	0·23
Blood	—	0·94	5	0·01
Magnesium	3·20	3·25	8	0·05
	1·60	1·60	2	—
+added Ca	1·60	1·62	5	0·02
Blood	—	0·63	6	0·01
Sodium	0·98	0·97	16	0·04
	1·96	2·00	15	0·04
	1·88	1·93	8	0·04
Blood	—	1·48	6	0·04
Blood+1 μg. Na	2·48	2·48	5	0·04

fact that the precipitate and the supernatant are similar in chemical composition and in density. It is possible that the accuracy might be improved by the adoption of a filtering technique rather than centrifugation for the separation of the precipitate. As the method stands, at least two estimations of sodium concentration must be made on each sample so that the error can be reduced to the same level as in the other cation measurements.

SUMMARY

A simple procedure is described for the estimation of sodium, potassium, calcium and magnesium in quantities of about 1 μg. The ions are precipitated by specific chemical reagents, the precipitates separated by centrifugation and converted quantitatively into chlorides. The chloride is titrated by the Volhard method or by an electrometric method using a simple automatic micro-burette.

Measurements made on simple salt solutions and on blood samples gave standard deviations of 1–2% except in the sodium method where the error was greater (2–4%).

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THE PERMEABILITY AND STRUCTURE OF THE CUTICLE OF THE AQUATIC LARVA OF *SIALIS LUTARIA*

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INTRODUCTION

In recent years a large number of investigations have been carried out on the structure of the insect cuticle, and this work has been thoroughly reviewed by Wigglesworth (1948). It has been the terrestrial insects, however, which have received most attention, and information on the structure of the cuticle of aquatic insects is still extremely limited. The work of Alexandrov (1935) on the permeability of the cuticle of *Chironomus* to certain water-soluble dyes suggests that, at least in this aquatic insect the permeability properties of the cuticle may be of a different nature from that of the terrestrial forms. On the other hand, observations by Wigglesworth (1933) on the swelling in tap water of mosquito larvae, ligatured near the posterior end, indicate clearly that in this species the cuticle is largely impermeable to water except in the region of the anal gills. Richards & Anderson (1942), studying the structure of the cuticle of this larva with the electron microscope, showed it to be two-layered, and the inner layer did not reveal the presence of any pore canals.

In terrestrial insects quantitative determinations have been made of the permeability of the cuticle to water by measuring the rate of evaporation of water through it (Ramsay, 1935; Wigglesworth, 1945; Beament, 1945), but no such facts are available for aquatic insects. Permeability of the integument to chloride might be estimated from data presented for the fall in blood chloride concentration after washing in distilled water as given by Wigglesworth (1938) for *Aedes* larvae and by Beadle & Shaw (1950) for *Sialis* larvae. In the former case the results will be greatly influenced by the presence of the anal gills which are areas of greater permeability than the rest of the cuticle.

The experiments described in this paper have been carried out to obtain quantitative data on the permeability of the cuticle of *Sialis* larvae to water and salts, and to attempt to correlate this information with the structure.

METHODS

The aquatic larvae of *Sialis lutaria* were collected locally and not selected for size or age. Their weights were measured on a 500 mg. torsion balance, reading to the nearest 0·5 mg., and ranged from 30–100 mg.

For measurements of the concentration of inorganic ions in the blood, a sample of blood was removed from a wound in the thorax by means of a micro-pipette, after

the larvae had been narcotized in water through which carbon dioxide was bubbled. The micro-pipette was made from a short length of 4 mm. glass tubing drawn out to a fine tip and blood was sucked up through the tip into the wider part of the tube. A very fine glass rod was introduced into the wider end of the pipette and the contained blood stirred until any clot which formed had collected on the stirrer, which was then removed. After sealing the tip in a small flame the blood could be centrifuged if necessary. Samples of supernatant were then removed by means of calibrated micro-pipettes for the purpose of analysis.

Chloride and sodium were determined by the methods described elsewhere (Shaw, 1955*a*). The volume used for each measurement was generally about 1 μl. and the error not greater than $\pm$ 2%.

For determinations of the permeability of the cuticle to water, heavy water (deuterium oxide) was used as a tracer, and the concentration of this substance in the blood was measured by estimating the density of a distillate prepared from the

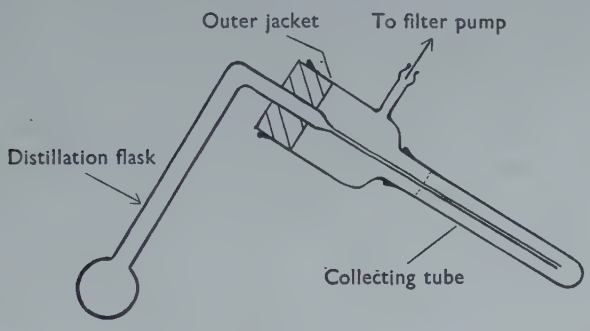

Fig. 1. Apparatus for the micro-distillation of blood samples containing heavy water.

blood. The density of deuterium oxide (1·10541 at 20° C.) is considerably greater than that of pure water and thus a density method provides a simple means of measuring its concentration.

The distillation was carried out on samples of blood which were collected by the method described above. The whole of the sample was transferred by means of a long narrow micro-pipette to the bottom of a small distillation flask made from a 10 cm. length of 5 mm. soda glass tubing, at the end of which a small bulb had been blown. With the sample inside the flask, the top was drawn out to a capillary and the neck bent at right angles just below this as shown in Fig. 1. The horizontal part of the neck fitted through the outer jacket so that the end of the capillary almost touched the end of the collecting tube. The collecting tube was connected to the outer jacket by means of an interchangeable ground glass joint, and the side tube of the jacket was connected to a filter pump so that the distillation could be carried out at reduced pressure. The bulb of the distillation flask was lowered into a boiling water-bath and the blood distilled until the whole of the water from the sample had passed over into the collecting tube. If some of the distillate stuck at the top of the capillary it could be sucked through into the collecting tube by the release and the subsequent re-application of the negative pressure.

In this way it was possible to collect 10 μl. of blood distillate, which is considerably more than is necessary for estimating the concentration of heavy water by the density method.

Measurement of the density of the sample was carried out by a simple modification of the gradient tube method described by Linderstrøm-Lang, Jacobsen & Johansen (1938). The gradient tube itself consisted of a 100 ml. graduated cylinder which stood in a water bath maintained at $20 \pm 0{\cdot}1^{\circ}$ C. 50 ml. of carbon tetrachloride were first added to the cylinder and 50 ml. of kerosene added on top of this. The interface between the two fluids was stirred rather vigorously with the flattened end of a narrow glass rod and the cylinder allowed to stand in the water-bath for several hours.

To make a determination, a small drop (a fraction of a micro-litre) of the blood distillate was introduced from a micro-pipette into the top of the cylinder and allowed to sink down until it reached its equilibrium position. The position of the drop in the cylinder was measured by means of a travelling microscope which was mounted vertically and could be read by a vernier to 0·02 mm. Measurements of both the top and the bottom of the drop were made and the mean of these two readings taken. This measurement was interpreted in terms of deuterium oxide concentration by introducing into the cylinder at the same time a series of calibrating drops containing known concentrations of heavy water. Because of the tendency for the density gradient in the cylinder to move its position over a period of time these calibrating drops were always added when a series of readings were taken. The density gradient for the concentrations used (between 0 and 20% D_2O) was reasonably linear and between 5 and 6 mm. in height. This allowed the concentration of heavy water in the drop to be estimated to within 0·1% D_2O.

For histological examination, the larvae were fixed in Bouin's solution, washed in 70% alcohol, dehydrated in dioxan, cleared and impregnated in celloidin by placing in a 1% solution of celloidin in methyl benzoate and finally embedded in paraffin wax. Sections were cut at 7μ and then stained either in haemalum and eosin or in Mallory's triple stain.

The thickness of the wax layer on the cuticle was estimated by utilizing the property of waxes to form a coherent monomolecular film when they are spread on a water/air interface. The cuticular wax can be extracted from a piece of cuticle of known area and spread on a clean water surface as a condensed monolayer and the area of the condensed film can be measured. On the assumption that the wax is evenly distributed over the piece of cuticle and that the wax molecules are orientated in the same manner in the cuticle as in the monolayer, the ratio of the monolayer area to area of the piece of cuticle from which the wax was derived gives the number of monolayers present in the cuticle. The thickness of the wax layer in the cuticle can then be calculated from an estimate of the average thickness of a monolayer of the wax.

In order to extract the wax from the cuticle, pieces of cuticle dissected as cleanly as possible from freshly killed larvae, were scraped clear of cellular material with a sharp scalpel and the inner side washed with petrol ether to remove any adhering

fatty material and blotted. The area of the piece was measured and it was introduced into a boiling tube made from a 5 cm. length of 4 mm. glass tubing, closed at one end. A small quantity of petrol ether was added and the other end of the tube was sealed off. The contents of the tube were heated by means of a small gas flame so that the petrol ether boiled and condensed at the cold end of the tube and in this way the piece of cuticle was refluxed in boiling solvent for 10 min. When cold the whole of the solvent together with washings was transferred by means of a micro-pipette to a Langmuir trough, which was similar in construction to that described by Adam (1938) except that it was much smaller. The trough measured $15 \times 5 \times 1$ cm. and was constructed from $\frac{1}{8}$ in. 'Perspex', and associated with this was a torsion balance which consisted of a float of waxed paper supported on a light glass capillary frame and suspended on a phosphor bronze torsion wire. The monolayer film was contained at one end of the trough by the float of the torsion balance and at the other by a glass slide ($3 \times \frac{1}{2}$ in.) coated with paraffin wax. The trough was filled with clean water and the petrol ether containing the cuticular wax was dropped on to the surface of this. When the ether had evaporated the remaining film was compressed with the glass slide until a sudden change in the reading of the torsion balance indicated that the film was fully compressed. At this point the area of the compressed film was measured.

PERMEABILITY TO WATER

Under normal conditions water enters the body of the larva continuously through the cuticle by osmotic uptake and a volume of water equal to this is expelled through the excretory system. There can be no great quantity of water stored in this system since daily records of weight of the larvae in tap water show little change over long periods of time. If a silk ligature is tied round the last segment of the abdomen then the weight of the larva increases, but this weight increase soon falls off and osmotic uptake of water must be prevented. This technique is not a reliable method for measuring water intake, and for accurate measurements heavy water was used as a tracer. In tracer studies with heavy water the flux of water molecules across the membrane is measured and not the osmotic uptake, but one can be calculated from the other. The method has the advantage that the larvae are not handled except at the end of the experiment and therefore there is no danger of damaging the cuticle by drying, etc. The only hazard that the larvae are exposed to during the experiments is the effect of the heavy water itself, but it is most unlikely that exposure to 20% deuterium oxide for maximum periods of one day would have any adverse effects on the animals. Indeed the larvae appeared quite healthy at the end of the treatment and would continue living in the heavy water solutions for very long periods.

For these experiments single larvae were placed in 3×1 in. specimen tubes each containing 5 ml. of a solution of 10 g. of Norsk Hydro 99·75% deuterium oxide made up to 50 ml. with glass-distilled water and the tubes were maintained at a constant temperature with a water-bath.

Larvae were kept in these tubes for periods of time varying from half an hour to one day and during these times were swimming about actively so that the solution

was kept effectively stirred. At the end of the period of treatment as much blood as possible was removed from the larvae and the concentration of heavy water in it was determined by the method described in the previous section. Measurements were carried out at two temperatures, at 20° C. which was just above room temperature and near the temperature at which much of the experimental work on *Sialis* larvae has been carried out (Shaw, 1955*b*), and also at a lower temperature, between 8 and 10° C. which is probably nearer the natural environmental temperature.

The results of the first series of experiments are shown in Fig. 2, where the blood concentration of heavy water is plotted against the length of time the larva had been in the 20% heavy water solution.

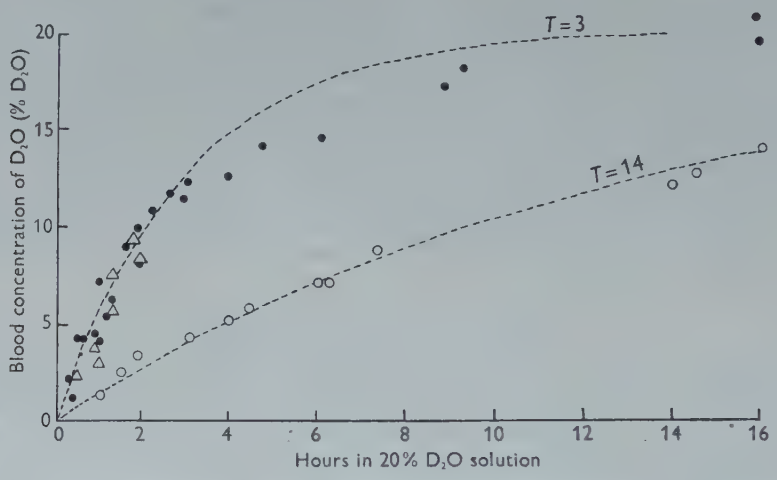

Fig. 2. The penetration of heavy water into larvae living in a 20% D_2O solution. ●, at 20° C. △, at 20°C, with necks ligatured; ○, at 8° C.

If the system of the blood separated from the outside fluid by the cuticle is considered as a simple physical one of a thin membrane separating two solutions which are well stirred (stirring of the blood will be brought about by its circulation) and if it is assumed that Fick's law can be applied to the diffusion of deuterium oxide molecules in water, then the internal concentration of heavy water in the blood will rise exponentially until it reaches that of the outside and the curve will have the form

$$C_i = C_o\{1 - \exp(-t/T)\},$$

where C_i = internal concentration and C_o = external concentration of deuterium oxide, t is the time and T is the 'time constant' for the penetration of D_2O which consists of two terms, the permeability constant (P) and the ratio of volume to surface area (v/s). Thus

$$T = v/Ps.$$

If t is in hours, v in ml. and s in cm.^{2}, then P is in cm./hr.

Since the ratio of the volume to the surface area in the specimens used is approximately constant, T can be used as a measure of the permeability of the membrane for comparative purposes.

In Fig. 2 two theoretical curves for $T=3$ and for $T=14$ are plotted on the same graph for comparison with the experimental results, and it is clearly seen that up to an internal concentration of 12% D_2O the experimental results conform to this type of function. Above 12% D_2O the experimental curve seems to flatten off and this may well be due to the establishment of a local concentration gradient on the inside of the cuticle because of insufficient stirring. With respect to this it must be noticed that if the major barrier to diffusion lies in the outermost layer of the cuticle then the inner part of the cuticle will form a layer where mixing will not readily occur.

The value of T which best fits the experimental points can be determined statistically over the range where these points follow the exponential, i.e. up to an internal concentration of 12% D_2O, by calculating the coefficient of the regression line of the logarithmic function

$$\ln (1-C_i/C_o)=-(1/T)t.$$

The coefficient is $-1/T$ and therefore T can be calculated:

At 8° C. regression coefficient $=-0{\cdot}0665$ and $T=15$.

At 20° C. regression coefficient $=-0{\cdot}329$ and $T=3$.

Since T is inversely proportional to the permeability at a given temperature, the ratio of these values gives the inverse ratio of the permeabilities at these temperatures. Thus

$$P_{(20°\,C.)}/P_{(8°\,C.)}=15/3=5,$$

and the Q_{10} for the permeability to water over this range of temperatures is 3·8. This is a high value for a Q_{10} but values as high as this are by no means unknown in permeability studies and the possible significance of this value is discussed later.

In order to be able to interpret these results in terms of cuticular permeability it was necessary to know if drinking occurred during these experiments. For this purpose the experiments at 20° C. were repeated with larvae whose necks were tied off tightly with silk ligatures and these results are also shown in Fig. 2. Although, as with the normal larvae, the results indicate a good deal of individual variation, the increase in internal concentration occurs at the same rate as in the normal larvae. Calculation of the regression coefficient as before gives a value of $T=3{\cdot}2$ and this value does not differ significantly from the value ($T=3$) for the normal larvae. Therefore, under the conditions of this experiment and probably under normal conditions with larvae in tap-water uptake of water through the gut does not occur.

The next series of experiments was designed to measure the flux of heavy water molecules moving from the blood outwards. There are some indications in the literature that cuticular permeability to water may be different in different directions. For example, Beament (1945) found that in the isolated cuticle of *Rhodnius* evaporation through the cuticle occurred 20 times faster when the inner side was exposed to the air.

For these experiments animals were kept in 20% deuterium oxide solution for 2 days at 20° C., and it was assumed that during this time the internal concentration had reached that of the outside. The larvae were removed and transferred either to

a large volume of distilled water at 20° C. or to running tap water at 10° C. At times varying between 1 and 14 hr. afterwards one of the larvae was removed and the heavy water concentration of its blood determined. The results of these experiments are shown in Fig. 3, and as in the previous figure some theoretical exponential curves are plotted on the same figure for comparison. In this case curves for $T=2$, 3, 8, 9 and 10 are plotted in the expression

$$C_i = C \exp(-t/T),$$

where C is the internal concentration at the start.

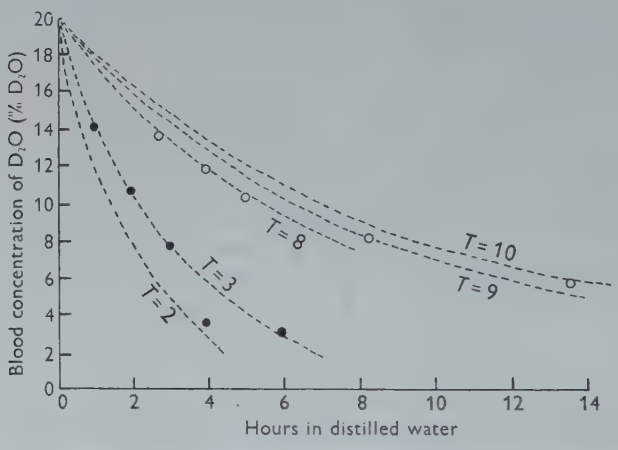

Fig. 3. The loss of heavy water from larvae transferred to distilled water after the blood concentration had been raised to 20%. ○, at 20° C.; and ●, at 10° C.

Not nearly as many determinations were made in this experiment as in the former, but the few experimental results tend to follow the expected type of exponential function, although there may be some flattening off in the curve for 10° C. There are not enough results for a statistical treatment, but inspection of Fig. 3 shows that all but one of the points for 20° C. lie on the curve for $T=3$ and at 10° C. between the curves for $T=8$ and $T=10$.

Thus it is not possible to detect any difference between the influx and outflux of deuterium oxide molecules across the cuticle at 20° C. At the lower temperatures a difference may exist since the average value for the outflux is $T=9$, whereas for the influx it is 11·4 (calculated from $T=15$ at 8° C.). Whether this difference reflects a real physiological difference or simply a difference in the experiments cannot be determined, but it may well be accounted for by differences in stirring. At 8° C. the animals are not nearly so active as at 20° C. and the heavy water solution was probably not adequately stirred, but on the other hand in the outflux experiments at 10° C. running tap water was used so that the stirring was very effective.

The Q_{10} for heavy water molecules diffusing outwards is approximately 3.

Finally, experiments were carried out to discover the rate of uptake of heavy water by the tissues when the larvae were placed in heavy water solutions. This information was necessary in order to estimate the effective volume of the larvae, a knowledge of which was required to calculate the permeability constant. It could also be used to measure the rate of exchange of water between the blood and the tissues.

The larvae were treated in the usual way in 20% heavy water solutions for periods of time varying between 30 min. and 2 hr. and then removed. They were weighed, then as much blood as possible was removed and they were weighed again. The concentration of heavy water in the extracted blood was measured. The larvae were finally transferred to a small flask made from a 3 cm. length of 4 mm. glass tubing at the end of which a small bulb had been blown and to the neck of this flask a longer piece of the same tubing, bent in the shape of a U, was fused. The bulb was introduced through the thermometer hole in the middle of the door of a small muffle furnace, and the temperature of the furnace was gradually raised until the temperature at the back was 200° C. During this time water distilled off from the animal, passed along the tube out of the oven and condensed and collected in the U portion of the tube. The apparatus was removed from the oven, the bulb opened and the larva was weighed again. Since the average dry weight of the larvae was known this last weighing served as a check that all the water had been driven off from the larva. The concentration of heavy water in the distillate was determined and compared with the concentration in the blood.

The results of these determinations are shown in Table 1. The values for blood concentration and tissue concentration are identical except in one case, and here the tissue is actually higher than that of the blood. This difference must be due to some contamination which has increased the density of the tissue sample.

Table 1. *A comparison of the concentration of heavy water in the blood and tissues of larvae kept in a 20% solution*

Time in 20% D_2O (hr.)	Blood conc. (% D_2O)	Tissue concn. (% D_2O)
0·5	5·1	5·8
1·12	4·3	4·3
1·25	5·3	5·3
1·4	6·3	6·3
1·9	11·2	11·2

A theoretical consideration of a system with two diffusion barriers such as is found here between the outside fluid and the blood and between the blood and the tissues, shows that as the concentration of heavy water in the blood increases the concentration in the tissues will also rise lagging behind the blood at first but will eventually catch up with it. The extent of the initial lag will depend on the ratio of the time constant for cuticle penetration to the time constant for cell membrane penetration. For example, if the outside concentration of heavy water is 20% and

the two time constants are equal, at the time at which the blood concentration had increased to 10% the intracellular concentration would be about half this.

The fact that no difference could be detected between the blood and tissue concentrations by the method used which will show differences of 0·1 % demonstrates that the time constant for the penetration of water into the cells is much smaller than that for the penetration through the cuticle by a factor of, at least, 20 and possibly much more.

By reason of the rapid exchange of water between the blood and the tissues the effective volume of the larvae will be the total body water which can easily be calculated from a knowledge of the wet and dry weights of the larvae. An analysis of nine larvae gave 81·3 ± 4·0 (S.D.) for the total body water expressed as a percentage of the wet weight.

The permeability of the cuticle can now be calculated for $T = v/Ps$ or permeability constant $P = v/Ts$. At 20° C., $T = 3$ and for a larva weighing 100 mg. the surface area $s = 1{\cdot}5$ cm.^{2} (approximately).

Thus $P = v/Ts = 81{\cdot}3/1000 \times 3 \times 1{\cdot}5 = 1{\cdot}8 \times 10^{-2}$ cm./hr. if it is assumed that the permeability of the cuticle is the same over the whole surface. The permeability constant, so derived, is for the penetration of D_2O molecules through the cuticle and is independent of the concentration of D_2O. There is no reason to suppose that the diffusion rate of H_2O molecules across the integument would be different from that of D_2O molecules, and thus the permeability constant is equally applicable to water.

This value for the permeability of the cuticle to water represents a very low order of permeability and it can be calculated from this figure that the diffusion rate of water molecules through the cuticle is in the order of 10^6 slower than it would be through a layer of water of the same thickness.

It is, however, not easy to compare the value directly with measurements of water permeability made on other animal membranes since few experiments have been made using heavy water as a tracer and thus flux values have not been measured. The experiments of Løvtrup & Pigoń (1951) on the penetration of heavy water into amoebae give a value of $P = 9 \times 10^{-2}$ cm./hr. for the permeability of the surface membrane of this animal which is not very different from the value derived for *Sialis* cuticle.

The vast majority of measurements of water permeability are of the osmotic uptake of water and are generally expressed as an amount of water crossing a given area of membrane in a given time for a known osmotic pressure difference. It is, however, possible to calculate the osmotic uptake of water from a knowledge of the flux values for water since the former is simply the difference between the water influx and outflux. The difference between these two is very small compared with the value of the flux itself and it is not possible from the type of experiments described above to separate true differences in flux values from differences due to experimental error and it is better to make the calculation in a more indirect way. The value for water flux through a membrane will be proportional to the activity (in the thermodynamical sense) of the water on the side of the membrane from which it is diffusing, so that if the ratio of activities of water on either side of the mem-

brane is known and also one of the flux values, then the other can be calculated and hence the difference between them.

Ussing (1952) has pointed out that this last relationship may not hold, and suggests a number of corrections that may have to be applied, particularly in cases where water forms a continuous phase in the membrane. However, in this case where the major barrier to diffusion must be the outermost wax layer (see below) these corrections are probably unnecessary.

Thus

$$\frac{\text{water influx } P_{\text{in}}}{\text{water outflux } P_{\text{out}}} = \frac{a_o}{a_i},$$

a_o and a_i are the activities of water on the outside and inside of the membrane respectively. Therefore

$$\frac{P_{\text{out}}}{P_{\text{in}}} = \frac{a_i}{a_o} \quad \text{and} \quad 1 - \frac{P_{\text{out}}}{P_{\text{in}}} = 1 - \frac{a_i}{a_o},$$

and

$$P_{\text{in}} - P_{\text{out}} = P_{\text{in}} \left(\frac{a_o - a_i}{a_o} \right),$$

Now $P_{\text{in}} - P_{\text{out}}$ is the osmotic uptake and the activities are proportional to the vapour pressures of the inside and outside solutions.

Therefore the osmotic uptake $(V) = P_{\text{in}}(p_o - p_i)/p_o$, and since the outer solution is practically pure water then by Raoult's law

$$V = P_{\text{in}} \frac{n}{n+N},$$

where n = number of solute molecules in inside fluid and N = number of solvent molecules.

Now the average vapour pressure of the blood has been measured by Beadle & Shaw (1950), and was found to be the same as a 1% NaCl solution which gives $n/(n+N) = 18/2925$.

Thus $V = 1{\cdot}8 \times 10^{-2} \times 18/2925$ cm.^{3}/cm.^{2}/hr.

$= 0{\cdot}11$ mg./cm.^{2}/hr. or $2{\cdot}64$ mg./cm.^{2}/day.

at 20° C.

The osmotic uptake of water at 20° C. of a larva weighing 100 mg. would be $2{\cdot}64 \times 1{\cdot}5 = 3{\cdot}96$ mg./day, or approximately 4% of the body weight per day. At 10° C., since the Q_{10} lies between 3 and 3·8, the water intake will be about 1% of the body weight per day and this value is probably nearer the uptake for larvae under natural conditions.

This value for the water uptake of 1% of the body weight per day is low compared with that found in many fresh-water animals, for example Krogh (1939) quotes figures of from 10 to 30% of the body weight per day for fresh-water fishes, and 25% for frogs. A higher value of 60% has been found for the earthworm, and from the values of urine production in *Anodonta* given by Picken (1937) a very high value of 600% can be calculated. Some fresh-water Crustacea appear to be less permeable, Krogh quotes figures for three species of fresh-water crayfish which vary between

3 and 5% but, on the other hand, *Daphnia* must be much more permeable to water (see Krogh, p. 96).

The osmotic uptake of water by the larvae of *Sialis* was also measured by a direct method. The accuracy of this method will not be so great as the heavy water method but it serves to test if the assumptions made in the derivation of the osmotic uptake from the measurements of the permeability constant of the cuticle are correct. The method used was to record weight changes that occurred in larvae with the excretory system blocked. As mentioned earlier, if larvae are ligatured near the posterior end swelling occurs but the rate of weight increase is not constant and it is not a satisfactory method of measuring water uptake. The method used was first to remove as large an amount of blood as possible from a larva through a small wound in the thorax. The wound was sealed by melting a small blob of 'Sira' adhesive wax over it and firm ligatures were tied round the neck and the last segment of the abdomen.

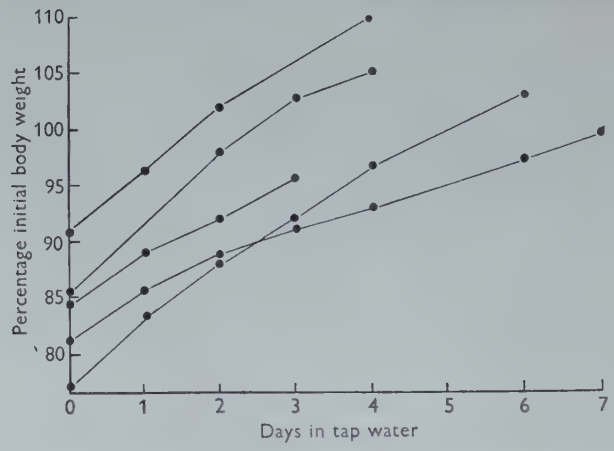

Fig. 4. The increase in weight of larvae replaced in tap water after removal of blood. Larvae prevented from drinking.

The larvae were weighed before and after the operation and replaced in tap water maintained at 20° C. in a water-bath. A daily record of the weight of the larvae was kept until the original body weight had been regained. In this way a constant increase in weight over a period of 2 or 3 days was observed. Fig. 4 shows the daily weight records of five larvae treated in this way and an analysis of the weight gains up to the time when the original weight is regained gives an average daily weight increase of 4·5% of the initial body weight per day and the range is from 2·7 to 5·8%. This is in very good agreement with the figure of 4% derived from the heavy water experiments.

PERMEABILITY OF THE GUT AND THE OCCURRENCE OF DRINKING

It was noticed, when carrying out the weight-gain experiments described above, that if the neck was not ligatured then the daily weight increases were much greater. This increased water uptake must be due to uptake of water through the gut and

the larvae must be drinking water under these conditions although normally, as has been shown earlier, drinking does not occur.

To measure this uptake larvae were treated in the same way as in the previous experiment, except that neither the neck nor the posterior end was ligatured. It was found in experiments reported elsewhere (Shaw, 1955*b*) that in larvae with the blood volume reduced the intake of water through the cuticle was still balanced by an equal output of water by the excretory system, so that if drinking is prevented no change in weight occurs in these larvae. In larvae where drinking is permitted, increases in weight must be due to water absorbed through the gut. Daily weight recordings were made of these larvae and the results of these experiments are shown in Fig. 5. It is clear that in every case a large amount of water is taken up during

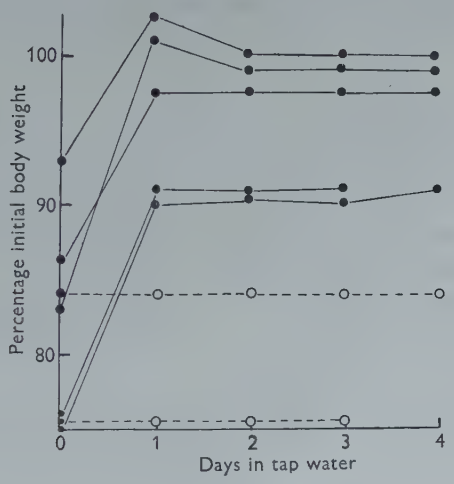

Fig. 5. The increase in weight of larvae replaced in tap water after blood removal. Drinking not prevented. ●, larvae in tap water; ○, larvae in isotonic mannitol.

the first day, an amount which is sufficient in some cases to restore the blood volume completely. After one day there is no further increase in weight which suggests that drinking ceases when the blood volume has been practically regenerated. The average value for the increase in weight during the first day is 13·1% of the initial body weight and the range from 8·2 to 18·9%. This is about 3 times greater than the osmotic uptake of water through the cuticle and the surface area of the gut will be smaller than that of the cuticle (by a factor of about ten) so that the permeability of the gut to water must be at least 30 times that of the cuticle. Since measurements of the weight increase were made at the end of the first day the possibility remains that the water might have been absorbed in a much shorter time so that this estimate for the permeability of the gut is a minimum one.

The fact that this absorption of water through the gut is due to osmotic uptake is demonstrated by experiments in which the larvae, after operation, are placed in an isotonic solution of mannitol. The results of two experiments, also shown in Fig. 5,

show no increase in weight so the possibility of an active transport of water under these circumstances is eliminated, although the solution is certainly swallowed.

The exact nature of the stimulus which causes drinking is not known but the fact that drinking does occur in larvae which have had a large amount of blood removed but the blood composition unaltered suggests that a reduction of either the blood volume or the blood pressure, or a combination of both, may be important factors.

It is interesting to notice that a similar increase in weight occurs in larvae which are moulting. In two cases where larvae were being weighed daily the animals moulted—up to the day of moulting the weight was constant, then during the day it increased markedly and then remained constant at the new level. The increase in weight in the two cases was 17·3 and 24·4% of the initial body weight respectively, and these figures are not very different from those found in the experiments just described. In fact, the stimulus for drinking may well be the same since an increase in the surface area of the cuticle will have the same effect as the reduction of blood volume and pressure.

PERMEABILITY TO INORGANIC IONS

Experiments on the washing of *Sialis* larvae in running distilled water or tap water (Beadle & Shaw, 1950) showed that chloride was only lost very slowly from the blood and that the larvae would stand many weeks of this treatment. The rate of loss of chloride from the blood was such that the blood chloride concentration fell in an approximately exponential manner with increasing time of washing. Further experiments were carried out in this way, at a measured and approximately constant temperature in order that the data could be treated in a quantitative manner and an estimate made of the permeability constant of the cuticle to chloride. The larvae were kept in isolation and were washed in slowly running tap water. The average temperature in the tubes occupied by the larvae was 14° C. and this was constant to ± 1° C. Measurements were made of the blood chloride and the blood sodium concentration after 3 weeks of this treatment, and the values were compared with the normal concentrations of these ions. The normal value for the chloride concentration is 31 mM./l. and this fell during the washing to a mean value of 12 mM./l. (S.D. ± 4 mM./l.) for six readings. Using the same equation that was applied to the outflux of water ($C_i = C \exp -t/T$) the time constant for the outflux of chloride was measured. At 14° C. this calculation gave $T = 20$ days.

This indicates that the permeability of the cuticle to chloride is considerably less than the permeability to water, a conclusion which is not unexpected.

The normal blood sodium concentration is much higher than that of the chloride —the average value is 109 mM./l. (Shaw, 1955*b*). After 3 weeks' washing the sodium concentration had fallen to 92 mM./l. (S.D. ± 7 mM./l.) (six readings), and the difference from the normal is highly significant ($t = 3{\cdot}3$ and $P < 0{\cdot}01$) at 17 mM./l.

Comparing the fall in the sodium level (17 mM./l.) with the fall in chloride level (19 mM./l.) under the same circumstances it appears that these two ions are being lost from the larvae at the same rate despite the threefold difference in their concentrations. Measurements have been made of the concentration of these two

ions in the excretory fluid, and the results are described in another paper (Shaw, 1955*b*). Chloride was not present in the fluid formed by larvae in tap water, and therefore the loss of chloride can be attributed wholly to diffusion through the cuticle. Low concentrations of sodium were found in the excretory fluid so the loss of sodium by diffusion through the cuticle may be even less than that of the chloride. These facts may indicate a difference in permeability of the cuticle to the two ions, but it is more probable that since the chloride ions are the only major diffusible anions in the blood, sodium is prevented from diffusing through the cuticle except in company with these anions. Indeed, the physiological function of the low blood chloride may be connected with this, since a high blood chloride would lead not only to a greater loss of chloride from the larvae under starvation conditions but also an increased loss of sodium and hence a substantial fall in total osmotic pressure of the blood.

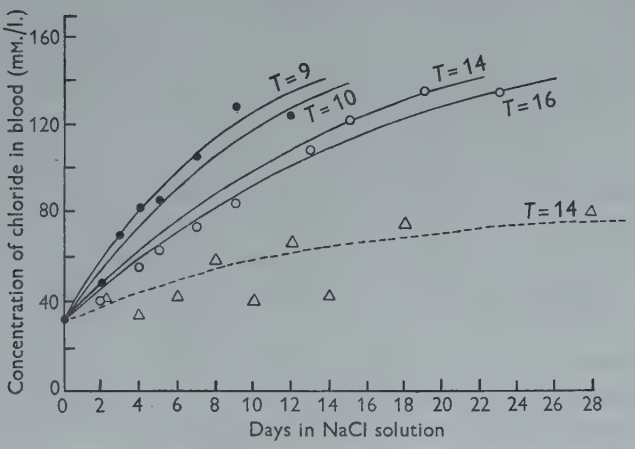

Fig. 6. The increase in blood chloride concentration of larvae living in NaCl solutions. ●, in 171 mM./l. NaCl at 23° C.; ○, in 171 mM./l. NaCl at 17° C.; and △, in 85 mM./l. NaCl at 17° C.

In order to see if any process other than that of passive diffusion through a relatively impermeable cuticle might be operating in the retention of chloride during prolonged washing, the rate of diffusion of chloride into the blood from external solutions of sodium chloride was also measured. If the loss of chloride during washing is a passive process then the permeability constants for the penetration of chloride in both directions would be expected to be the same.

Larvae, with their necks ligatured to prevent uptake of the outside fluid via the gut, were placed in solutions of sodium chloride of known concentrations and the blood chloride measured after intervals of time varying from 2 to 28 days. The results of these experiments are shown in Fig. 6 which illustrates the changes in blood chloride which occurred in larvae kept in 85 mM./l. NaCl at 17° C. and in 171 mM./l. NaCl at 17° C. and at 23° C.

The interpretation of these results, in terms of a permeability constant for chloride, is complicated by the difference in concentration of sodium and chloride

in the blood. The rate of penetration of chloride through the cuticle cannot be governed entirely by the difference between the outside and inside concentrations of sodium since penetration of chloride occurs, at least in some larvae, from 85 mM./l. NaCl where the external sodium concentration is actually lower than that of the blood. It is possible that here the chloride is exchanged for some other anion in the blood. In the case of penetration of chloride from the 171 mM./l. NaCl solution, both diffusion of sodium chloride and this anion exchange will probably be taking place.

The time constant for the penetration of chloride into the larvae was estimated by using the equation

$$C_i - C = (C_o - C)\{1 - \exp(-t/T)\},$$

where C is the initial blood Cl concentration; C_i is the blood concentration after time, t; and C_o is the outside Cl concentration.

In view of the above considerations this equation will give a maximum value for the time constant.

In Fig. 6 curves are plotted for different values of T with C_o equal to 171 mM./l. for comparison with the experimental points. The latter follow the expected type of exponential function and for penetration from 171 mM./l. solutions the time constant (T) lies between 9 and 10 days at 20° C. and between 14 and 17 days at 17° C. From the 85 mM./l. solutions the scatter is much greater; in some larvae the blood chloride has not risen at all, but in others a time constant of about 14 days is found.

The values of the time constant for the outflux of chloride at 14° C. ($T = 20$) and for the influx of chloride at 17° C. ($T = c.\ 15$) are not sufficiently different, considering the dissimilar temperatures, to indicate a real difference in permeability of the cuticle in opposing directions. However, since the value of T for the influx is a maximum the possibility of such a difference cannot be completely excluded. It could be checked by using radio-active ions as tracers.

The permeability constant of the cuticle to chloride can be measured from a knowledge of the time constant, using the relation $P = v/Ts$. In this case v = the blood volume which is 56% of the body weight (Beadle & Shaw, 1950); $s = 1{\cdot}5$ cm.^{2} for a larva weighing 100 mg. as before.

Thus at 17° C.

$$P = v/Ts = 56/1000 \times 1{\cdot}5 \times 15 \times 24$$
$$= 1{\cdot}04 \times 10^{-4} \text{ cm./hr.}$$

Comparative figures for the permeability of other animal membranes to chloride are not available. However, this figure can be compared with similar constants derived from the penetration of sodium and potassium. Krogh (1946) lists values for a number of membranes including plant cells, red blood corpuscles and muscle cells and these range from 6×10^{-7} to 7×10^{-4}.

THE STRUCTURE OF THE CUTICLE

An examination of histological sections of larvae prepared from different regions of the body and stained with Mallory's triple stain, showed that the cuticle consisted of at least two distinct layers. The innermost layer, which stained brightly blue and showed evidence of horizontal layering was clearly homologous with the endocuticle of terrestrial insects (see Wigglesworth, 1948). This layer was covered on the outer side by a thinner epicuticle which was sharply differentiated from the endocuticle and stained red. In the abdomen where the cuticle is quite transparent—the natural dark colour is due to pigment granules in the epidermal cells—these are the only two layers which can be identified by microscopical examination of the sections. In the regions of the head and thorax the cuticle is much harder than the soft abdominal cuticle, and this is partly due to the fact that the epicuticle is further differentiated so that it comprises two distinct layers. The inner of these resembles the epicuticle of the abdomen in staining properties but the outer, which is thinner, is naturally amber in colour and in sections treated with ammoniacal silver nitrate solution gives a positive argentaffin reaction. This presumably is a region of polyphenol-tanned protein (Pryor, 1940; Wigglesworth, 1948) and will be referred to as the polyphenol layer. The epicuticle of the abdomen and of the abdominal gills never gives this reaction and this polyphenol layer must be absent. Where the layer is present it is found only in the outer region of the epicuticle and there is no indication of any layer equivalent to the exocuticle of some other insects.

Average values for the thickness of these cuticular layers in different regions are given in Table 2. The total thickness of the cuticle of the abdomen is considerably less than that of the thorax and the abdominal gills are covered with a cuticle which is only slightly thinner than that over the rest of the abdomen. In the histological sections it was not possible to detect the presence, outside the layers just described, of any layer corresponding to the wax-containing outer epicuticle of many other insects, but in any case its thickness is likely to be below that which could be resolved by the microscope. The presence of a wax layer was, however, indicated by some indirect observations. The cuticle, for example, has a waxy-like sheen and is not easily wetted by water after the larvae have been removed from water. More substantial evidence comes from the fact that larvae will live for many hours in a solution of ammoniacal silver nitrate as shown by Wigglesworth (1945) for some terrestrial insects, and during this time there is no appreciable darkening of the

Table 2. *The thicknesses of the various layers of the cuticle of* Sialis *larva from different regions of the body*

Layer	Thorax (μ)	Abdomen (μ)	Abdominal gills (μ)
Endocuticle	20	7	5
Epicuticle	6	1	1
Polyphenol layer	1	Absent	Absent
Wax layer	—	0·1	0·1

polyphenol layer. This must mean that this layer is covered over by some other layer which is impermeable to this solution. If the larvae are allowed to walk about in alumina powder (Wigglesworth, 1945) for 2 min. and then placed in the silver nitrate solution they die in 2 hr. showing some blackening at the joints of the legs and the gills. If the treatment in alumina lasts for 15 min. then death will occur in the silver nitrate solution in a few minutes with much more general blackening. The fact that blackening occurs after this treatment shows that this thin layer has been rubbed away. Now it has been conclusively demonstrated in the terrestrial insects (Wigglesworth, 1945; Beament, 1945) that it is this outer thin layer which is very largely responsible for the low permeability of these insects to water and that at least part of this layer consists of a continuous film of wax. That wax is also present in the cuticle of *Sialis* larvae was demonstrated by the extraction of such a material from either pieces of fresh cuticle or from cast skins by the application of hot organic

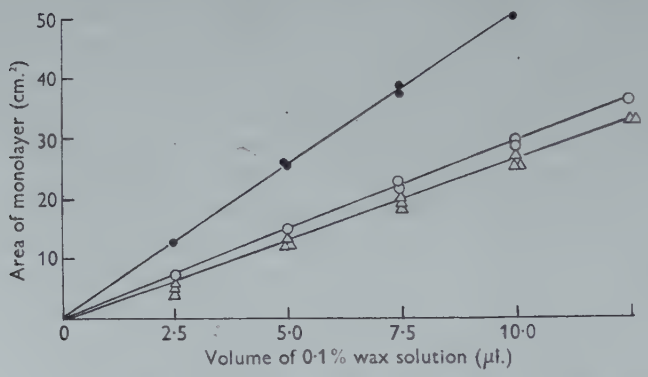

Fig. 7. The areas of known amounts of lipoids spread on the trough. ● is palmitic acid, ○ is beeswax, and △ is locust wax.

solvents such as chloroform or petrol ether, and it was found that this extracted wax could be spread on the surface of clean water to give a condensed monolayer. This fact was made use of in determining the thickness as described in an earlier section.

The small Langmuir trough was first calibrated by spreading on the water surface known quantities of palmitic acid from a petrol ether solution. The films were compressed with a waxed slide until the surface balance started to indicate a steep rise in the surface pressure, and the area of the film was measured at this point. The largest area that could be measured on this trough was about 50 cm.^{2} and a film of this area is given by 10 μg. of palmitic acid so the method is a very sensitive one. A solution of beeswax in petrol ether of the same concentration (0·1 %) was also used, and it was found that beeswax gave a stable monolayer but the area of the condensed film was not so constant as for palmitic acid. A purified preparation was made of the cuticular wax from the cast skins of locusts by extracting these skins with boiling petrol ether, evaporating the solvent and dissolving in petrol ether again to give a 0·1 % solution. Small quantities of this solution were also spread on

the trough, and the area of the locust wax monolayer compared with that obtained for the beeswax. Fig. 7 shows the results obtained for the three substances, palmitic acid, beeswax and locust wax. For the same volume of solution the two waxes give films of very nearly the same size and these are only about half the area of the film given by the same volume of palmitic acid solution. For the same weight of palmitic acid and beeswax the ratio of the areas of the condensed films is 30/17 and the ratio of specific gravities is 0·85/0·96. From these figures it can be seen that the ratio of the thickness of the two films is $30 \times 0{\cdot}85/17 \times 0{\cdot}96 = 1{\cdot}54$. Now the palmitic acid molecule consists of a straight chain of 16 carbon atoms so that if the beeswax molecules are also straight chains then the average number of carbon atoms in a molecule is $1{\cdot}54 \times 16 = 25$. If the specific gravity of the locust wax is assumed to be the same as that of beeswax then applying the same argument the number of carbon atoms in this case would be 26. This estimate agrees with the published accounts of the composition of insect waxes as determined by chemical methods. For example, Chibnall, Piper, Pollard, Williams & Sahai (1934) and Bergmann (1938) describe waxes containing mixtures of acids, esters and paraffins of chain length between C_{26} and C_{31}.

The thickness of a film of locust wax can easily be calculated since the C—C bond distance in a straight hydrocarbon chain is 0·143 mμ; $26 \times 0{\cdot}143 = 3{\cdot}7$ mμ.

Table 3. *The estimated thickness of the wax layer on pieces of cuticle from different body regions of* Sialis *larva and locust nymph*

	Area of piece of cuticle (mm.²)	No. of monolayers	Thickness of wax layer (μ)
Sialis			
Abdomen	91	26	0·09
	90	59	0·20
	75	30	0·11
	54	30	0·11
	72	22	0·08
	50	26	0·09
			Mean 0·11
Gills	35	43	0·16
	35	22	0·08
	32	43	0·16
			Mean 0·13
Locust			
Top of femur	50	100	0·37
	50	87	0·32
	50	92	0·34
			Mean 0·34
Prothorax	20	94	0·35
	52	74	0·27
	31	101	0·37
			Mean 0·33
Abdomen	83	62	0·23
	78	65	0·24
	85	70	0·26
			Mean 0·24

These calculations have been carried out for locust wax since this was easy to obtain and prepare, but no attempt has been made to collect appreciable quantities of *Sialis* wax and it has been assumed that the thickness of a film of this wax will be the same as that from the locust. The thickness of the cuticular layer was estimated by comparing the area of a piece of cuticle with the area of the wax monolayer film obtained from it. The ratio of these two areas multiplied by the estimated thickness of each film (3·7 mμ) gives the thickness of the wax layer. This method is quite a delicate one and small pieces of cuticle, between 20 and 100 mm.^{2} can be used for each determination.

Table 3 shows the results of determinations made in this way on pieces of cuticle from the abdomen of *Sialis* larvae and from the tracheal gills which were freed as much as possible from material other than cuticle. Measurements were also made on pieces of cuticle from the locust so that a comparison could be made with a terrestrial insect. The average values (between 0·24 and 0·34 μ) for different regions of the locust cuticle are very similar to those values deduced by other means for terrestrial insects (Beament, 1945). The figures for *Sialis* show that the wax layer is 2–3 times thinner than that of the locust, but it is interesting to find that the layer on the abdominal gills is about the same thickness as that elsewhere on the abdomen.

DISCUSSION

The permeability constant for the diffusion of water through the larval cuticle of *Sialis* ($P = 1{\cdot}8 \times 10^{-2}$ cm./hr.) as determined by the heavy water experiments suggests a low order of permeability, and it is interesting to compare the permeability of this membrane with that of the various cell membranes which have been measured. Lucké, Hartline & Ricca (1939) summarize the results of many determinations of water permeability on a variety of cells, including invertebrate egg cells, Protozoa, plant cells and vertebrate blood cells. The results are expressed as an osmotic uptake in μ^3/μ^2/min./atmosphere difference in osmotic pressure. From the derived osmotic uptake in *Sialis* (0·11 mg./cm.^{2}/hr.) the water penetration can be calculated in these units. The average value for the cell membranes is 0·6 with a range from 0·1 to 3·0, but for *Sialis* larval cuticle the uptake is only $1{\cdot}8 \times 10^{-3}$ and therefore this membrane is some 300 times less permeable than the average for the cell membranes. This difference may be due in part to the fact that the wax layer, which is the major diffusion barrier, is considerably thicker than the average for the lipoid part of the cell membrane (100 mμ as against 10 mμ) but must also reflect the different physical properties of cuticular wax as compared with membrane lipoids.

However, some cell membranes may have a much lower permeability, for example the determinations of Løvtrup & Pigoń on *Amoeba* indicated a permeabilty only 5 times that of *Sialis* and measurements on the trout egg (Gray, 1932; Krogh & Ussing, 1937) suggest that the membrane around this cell may be completely impermeable to water.

To compare the permeability of the larval cuticle of *Sialis* with that of other insects is not so easy because no measurements of permeability constant or of osmotic

uptake of water have been made. A large number of measurements have been made of the evaporation rate from terrestrial insects (Ramsay, 1935; Wigglesworth, 1945), but it is not possible to compare these rates directly with the water outflow as determined in *Sialis*, since two factors determine the evaporation rate. First, the rate will be an expression of the number of molecules crossing the membrane (which might be comparable with the outflux with water on both sides), and secondly the rate of diffusion of water molecules away from the surface of the membrane. In the case of a free water surface it will be the rate of diffusion from the surface which is the limiting factor. In order to make a comparison possible a few measurements were made of the rate of evaporation of water from *Sialis* larvae suspended in air.

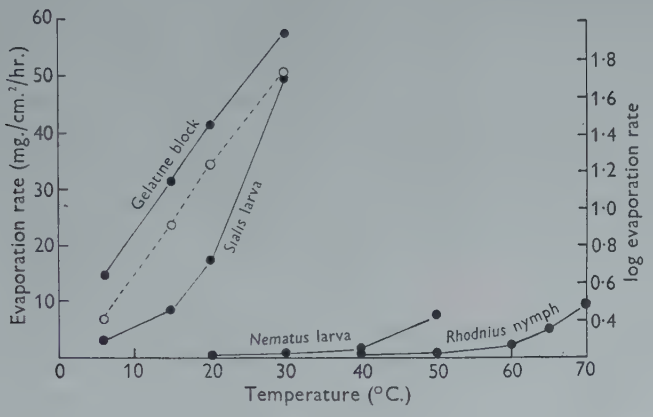

Fig. 8. The evaporation rate of water from *Sialis* larvae compared with some terrestrial insects. ○ are points representing the logarithm of the evaporation rate for *Sialis* larvae at the same temperatures.

The apparatus was modelled as closely as possible on that described by Wigglesworth (1945), the drying agent was contained at the bottom of a conical flask and the animals were suspended by threads tied round their necks so that they hung just above the surface of the drying agent. Controls representing a free-water surface were made by suspending in a similar manner weighed blocks cut from a 20% gelatine gel and roughly the same shape and weight as the larvae.

Fig. 8 shows the results of these experiments indicating the loss of weight per hour through a surface of 1 cm.² at different temperatures and in the same figure values for *Nematus* larva and *Rhodnius* nymph, taken from Wigglesworth's paper (1945, fig. 1, p. 98), are inserted for comparison. The rate of loss of water from *Sialis* larvae is very much greater than that from the two terrestrial insects; at 30° C. it is about 100 times greater than the loss from *Nematus* larva at the same temperature. There appears to be very little difference between the evaporation rate from *Sialis* larvae and the gelatine block, particularly at the higher temperatures and since it has already been established that with an aqueous medium on either side of the membrane the cuticle presents a very considerable barrier to the penetration of

water, this appears at first to be something of a paradox. The explanation lies in the relative importance of the two processes which together determine the evaporation rate. From a free water surface it is the diffusion of molecules away from the surface which is the limiting factor; thus at 15° C. it can be calculated from the vapour pressure that the mass of water leaving 1 cm.^{2} of surface in an hour would be 560 g., but it is seen from Fig. 8 that the evaporation rate is only 31 mg/cm.^{2}/hr. Therefore less than 1 water molecule in 10,000 of those which initially leave the surface permanently escapes under these particular conditions. On the other hand, the heavy water experiments on *Sialis* larvae showed that the mass of water passing through the cuticle in one direction is 18 mg./cm.^{2}/hr. at 20° C. (p. 335) and it is assumed that the same will be true of the larva suspended in air. From Fig. 8 the evaporation rate is seen to be 17 mg./cm.^{2}/hr. at 20° C., which is almost exactly the same, and therefore in this case it is truly the rate of passage of molecules across the surface film and not the subsequent diffusion rate which is measured. The high Q_{10} for the evaporation rate from *Sialis* larvae (between 3 and 4, cf. value measured by heavy water experiments, p. 338) compared with the value of 1·3 for the water surface confirms that different processes are being measured in the two cases. The figures given by Wigglesworth for *Nematus* larva and *Rhodnius* nymph also indicate very high Q_{10} values, and there is little doubt that the same argument is applicable to these other insects.

Thus although the rate of evaporation from an insect may appear to approximate to that of a free water surface a difference in permeability of at least 10^4 may be disguised by this type of measurement.

It is interesting to see if the limited data shown in Fig. 8 for the evaporation rate from *Sialis* larvae reveal a transition in the rate of water loss occurring at a 'critical temperature', such as has been described for many terrestrial insects (Ramsay, 1935; Wigglesworth, 1945). It was seen above that the temperature coefficient (Q_{10}) for the evaporation rate was very high and it is this fact that accounts for the characteristic shape of the evaporation rate curves. Now if the Q_{10} of any process is constant over a range of temperatures, then the rate at which the process occurs will vary exponentially with the temperature, or in other words the logarithm of the rate will be porportional to the temperature. If a sudden transition occurs in the evaporation rate at a particular temperature then the Q_{10} for the range of temperatures including this one will reflect this change and the curve relating the logarithm of the evaporation rate to temperature will show a sudden change of slope at this point.

This logarithmic relation has also been plotted with the data for *Sialis* larvae (Fig. 8, dotted line), and is seen to be a more or less linear one. Thus the Q_{10} is relatively constant over the temperature range studied (6–30° C.) and there is no reason to postulate any discontinuous change in the permeability properties of the cuticle with increasing temperature. However, owing to the small number of measurements, the possibility cannot be completely excluded but it would be more satisfactorily tested by the application of the D_2O tracer method for the determination of the permeability constant to water over a wide range of temperatures.

Since the thickness of the wax layer in *Sialis* larvae is not more than 2 or 3 times less than that of many terrestrial insects, the very large differences in permeability of their cuticles to water must be due to differences in the chemical and physical nature of the waxes.

Despite the fact that the permeability of the cuticle of *Sialis* larvae is greater than that of most terrestrial insects, it is still very low compared with the body surfaces of the majority of fresh-water animals and as a result, compared with these animals, water intake and salt losses are low, although the surface area of the body is increased by the presence of abdominal gills. This low permeability of the cuticle is probably of wide occurrence among aquatic insects and a few preliminary readings made on the wax extracted from the cuticles of the larvae of *Aeschna cyanea*, *Ephemera danica* and *Anabolia nervosa* suggest that the thickness of this layer is not very different from that found in *Sialis*, the values ranged from 0·08–0·15 μ.

Many aquatic insect larvae possess tracheal gills attached to the abdomen and these may well resemble those of *Sialis* in possessing a thin layer of wax over the surface. This layer will not greatly resist the diffusion of oxygen, although the resistance to water and salt movement is very high. In this way, these gills will greatly increase the surface available for respiratory exchange without affecting the salt loss very much.

SUMMARY

1. The permeability to water of the cuticle of *Sialis* larvae has been measured, using heavy water as tracer. The penetration was slow, the permeability constant being only $1{\cdot}8 \times 10^{-2}$ cm./hr. at 20° C. There was no obvious difference between the rate of water influx and outflux. The rate at which water penetrated into the tissues from the blood was much greater than through the cuticle. The Q_{10} for diffusion through the body surface was high, lying between 3·0 and 3·8. The osmotic uptake of water was calculated to be about 1 % of the body weight per day at 10° C.

2. Drinking of water did not occur in normal larvae, but in larvae with the blood volume reduced, osmotic uptake of water through the gut did take place and the gut wall was much more permeable to water than the cuticle. A similar intake of water probably occurred during moulting.

3. The permeability of the cuticle to chloride was measured and also found to be of a low order ($P = 1{\cdot}04 \times 10^{-4}$ cm./hr. at 17° C.). Sodium diffused out of the larva at the same rate as the chloride.

4. Histological examination of the cuticle showed that in the abdomen it was thin and consisted of a 7 μ thick endocuticle and a 1 μ epicuticle. Over the thorax it was thicker, and a polyphenol layer was present as the outer layer of the epicuticle. There was indirect evidence of the presence of a wax layer.

5. Wax was extracted from the cuticle, and the thickness of the layer from which it was derived was estimated by means of a monolayer technique. In the cuticle of the abdomen and gills the thickness averaged 0·1 μ.

6. The permeability to water of the cuticle was compared with that of terrestrial insects and was found to be much greater. This difference was not due to the thick-

ness of the wax layer but probably to some physical properties of the wax. The cuticle of *Sialis* larvae showed no 'critical temperature' or sudden change in the permeability properties with temperature over the range of temperatures studied.

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IONIC REGULATION AND WATER BALANCE IN THE AQUATIC LARVA OF *SIALIS LUTARIA*

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INTRODUCTION

The work described in this paper is a continuation of the investigations on the osmotic and ionic regulation of *Sialis* larvae reported by Beadle & Shaw (1950). In this work it was found that the larvae possessed no mechanism for the absorption of ions from dilute solutions, as have mosquito larvae, for example (Koch, 1938), and that chloride was retained in the larvae by the very low permeability of the cuticle. Further, variations in the chloride content of the blood were not paralleled by variations in the total osmotic pressure, and measurements of the non-protein nitrogen content of the blood suggested that changes in the salt content in the blood were compensated by changes in the concentration of the blood amino-acids. At that time very little was known of the regulatory powers of aquatic insects with respect to the blood inorganic ions and, in fact, the composition of the blood had not been determined in detail. Wigglesworth (1938) had found that in the mosquito larva, kept in saline solutions of less than the blood concentration, maintained the blood chloride at its normal level so that some regulatory mechanism appeared to be at work.

The experiments in this paper were carried out in order to throw further light on the normal blood composition of *Sialis* larvae and to discover how both the normal concentration of inorganic ions in the blood and the normal water content of the larvae are maintained.

Recently the knowledge of ionic regulation in the mosquito larva has been greatly advanced by the work of Ramsay (1951, 1953*a*), who has described the role of the Malpighian tubules and the rectum in regulating the potassium and sodium content of the larvae, using an elegant micro-flame photometric technique.

As far as ionic regulation and water relations are concerned, *Sialis* larvae differ in a number of important ways from mosquito larvae. Thus *Sialis* larvae possess no ion-absorbing mechanism, and as a consequence there is no region of the cuticle that is especially permeable to water, so water penetration is slow (Shaw, 1955*b*). In the excretory system there is no circulation of fluid into the mid-gut, as far as is known, and the excretory fluid is itself not much less concentrated than the blood.

It is, therefore, of considerable interest to compare the regulatory mechanisms found in *Sialis* with similar mechanisms in the mosquito larvae, where this is possible.

METHODS

Samples of blood for the quantitative analysis of its inorganic constituents were collected in the manner described in a previous paper (Shaw, 1955*b*). On the extracted blood and on the excretory fluid estimations were made of the concentrations of sodium, potassium, calcium, magnesium, chloride and bicarbonate.

The four cations and chloride were estimated by means of the ultra-micro methods described in another paper (Shaw, 1955*a*).

Bicarbonate was determined by liberating carbon dioxide from the sample by the addition of acid and absorbing the CO_2 in standard alkali. This reaction was carried out in a micro-diffusion cell of the type described by Shaw & Beadle (1949): the gas was liberated by the addition of 50 μl. of N sulphuric acid and absorbed in a 5 μl. hanging drop of N/20-NaOH containing phenolphthalein as indicator. The excess alkali was titrated with 0·05 N-HCl using the titration apparatus described by Shaw (1955*a*). The time allowed for the diffusion was 2 hr. and the sample used for each determination contained between 5 and 10 μg. of bicarbonate.

Measurements of conductivity were made with the aid of a Mullard conductivity bridge and a conductivity cell which contained 2 ml. of fluid. The sample (about 1 μl.) was diluted to this volume with glass-distilled water and the bridge and cell was calibrated empirically with standard sodium chloride solutions prepared in the same way. At this dilution the accuracy was not greater than $\pm$ 10 mM./l. NaCl.

Membrane potentials were recorded by means of a valve millivoltmeter consisting of a pair of RCA 954 valves connected as electrometers in a balanced bridge circuit. The input impedance was 100 MΩ and the measurements were made to the nearest 0·5 mV.

BLOOD COMPOSITION

The total osmotic pressure of the blood of *Sialis* larvae has been measured by Beadle & Shaw (1950), who found it equivalent to the osmotic pressure exerted by a 1% sodium chloride solution approximately. This value for the osmotic pressure of the blood is one which is found in many fresh-water and terrestrial animals (see Krogh, 1939), but generally the solute particles which give rise to this osmotic pressure consist largely of sodium and chloride ions. This is not usually the case in insects; thus Beadle & Shaw found an average chloride ion concentration of 34 mM./l. in *Sialis* larvae, and this would account for only 10% of the total osmotic pressure. Similarly, Wigglesworth (1938) had found that in the mosquito larva in tap water the chloride ion contribution was about 18%. It was suggested, as a result of the large amount of non-protein nitrogen that was found in the blood of *Sialis* that the presence of free amino-acids might account for much of the osmotic pressure deficit. In order to have a clearer understanding of the relative contribution of inorganic ions to the total osmotic pressure of the blood a more detailed analysis has been made, and the results are shown in Table 1.

The sodium concentration (109 mM./l.) is much higher than that of the chloride and accounts for some 32% of the osmotic pressure. This is similar to recent measurements made on the mosquito larva by Ramsay (1951, 1953*a*), who found an

average concentration of 108 mM./l. for sodium in the blood of larvae kept in distilled water and, as in this case the total osmotic pressure was lower than that of *Sialis* blood, the sodium ions accounted for 42% of the total.

Table 1. *Blood composition*

Measurement	No. of readings	Concentration		S.D. (±mM./l.)
		mM./l.	m.equiv./l.	
Total O.P. as an isotonic sucrose soln.	31	339	—	26
Conductivity as equiv. NaCl soln.	7	152	152	17
Sodium	21	109	109	10
Potassium	10	5	5	1·5
Calcium	6	7·5	15	4
Magnesium	6	19	38	4
Sum of cations	—	140·5	167	—
Chloride	19	31	31	10
Bicarbonate	4	15	15	—
Sum of anions	—	46	46	—
Total ionic concentration		186·5	213	—

The total concentration of anions and cations which have been measured is 186·5 mM./l. and this would give rise to an osmotic pressure of only 55% of that found leaving a deficit of 152·5 mM./l. The sum of the cations—and these are probably the only ones present in any quantity—is 167 m.equiv./l. and this is very similar to the value of 152 m.equiv./l. determined from the conductivity measurements. Therefore the great majority of the cations must be present in an ionized form. This is, however, a big discrepancy in the concentration of anions as measured and as predicted from the conductivity measurements. The sum of the anions (46 m.equiv./l.) is less than one-third of the value of 167 m.equiv./l. required by the conductivity. The blood must contain, therefore, a large unknown anion fraction to which other inorganic anions such as phosphate and sulphate are unlikely to contribute very much.

It is not possible to calculate what fraction of the total osmotic pressure deficit of 152·5 mM./l. is made up of these unknown anions since their valency is not known. If the valency of these anions was one then they would contribute some 70% to this deficit and the total electrolyte content of the blood would account for about 90% of the total osmotic pressure. If the average valency was two, then the contribution to the deficit would only be 35% and so on.

Proteins in solution often behave as polyvalent anions and their effect can be considered since the average concentration in the blood is known (Beadle & Shaw, 1950) to be 5·2%. This amount would have a negligible osmotic effect but could exert an effect equivalent to 5–10 m.equiv./l. on the conductivity, but this is small compared with the 121 m.equiv./l. that still has to be accounted for.

It was also found by Beadle & Shaw that the blood concentration of non-protein nitrogen-containing molecules (av. 258 mg. N%) was such that if each molecule contained 1 atom of nitrogen the concentration of these molecules would be 184 mM./l. This is greater than the osmotic deficit, and therefore some of these

molecules must contain more than one molecule of nitrogen and might be peptides. Owing to the large amount of these nitrogen-containing compounds which are present, it is probable that the unknown anions form part of this fraction and therefore might consist of dicarboxylic amino-acids or peptides containing these.

The potassium concentration is quite low and characteristic of the carnivorous insects (Boné, 1944) and similar to the concentration found in other aquatic insects. Thus Ramsay (1953*b*) gives values of blood potassium in *Aedes*, *Dytiscus* and an aquatic Tabanid as 3, 5 and 5 mM./l. respectively. Both the divalent cations, calcium and magnesium, are present in high concentrations and the magnesium is especially high, the concentration approaching that found in the marine crabs. However, the high concentrations of these ions and the low concentration of chloride ions seem to be characteristic features of insect blood composition.

INORGANIC CONSTITUENTS OF THE TISSUES

Measurements of the total amounts of some of the inorganic constituents of *Sialis* larvae indicated that the distribution in the tissues must be very different from that in the blood. Beadle & Shaw (1950) measured the total chloride of the larvae and showed that if the chloride was confined entirely to the blood then the blood volume was approximately 56% of the wet weight of the larvae. This estimate was confirmed by blood-dilution experiments. From a knowledge of the blood volume and of the blood concentration of the inorganic ions it is possible to calculate their concentration in the tissues, and experiments were carried out to determine the tissue concentrations of the four cations.

A larva was first weighed (W_1), then as much blood as possible removed and the larva weighed again (W_2). The dry weight was determined by heating to constant weight at 100° C. (W_3). The dried larva was incinerated in a small crucible at 450° C. for several hours until the organic material had been removed, the resultant ash dissolved in 50 μl. of N/5-HCl and the concentration of the cations in this solution measured. The concentration of cations in the blood sample which had been removed was also determined.

Now the total weight of water in the larva $= W_1 - W_3$ mg. and the blood weight $= 0{\cdot}56W_1$ and therefore the weight of water in the tissues $= (W_1 - W_3) - 0{\cdot}56W_1 = T$. Now the weight of blood in the larva after the blood extraction will be $W_2 - W_3 - T = B$. If C_1 = concn. of the ion in the blood in mM./l.; C_2 = concn. in mM./l. in the 50 μl. N/5-HCl and C_3 = concn. of the ion in the tissues in mM./kg. tissue water, then

$$50C_2 - BC_1 = TC_3,$$

so that C_3 can be calculated.

Table 2 shows the results of these calculations for the four cations. Potassium replaces sodium as the most important ion; the calcium concentration is not very different from that of the blood but the magnesium is much higher. Nothing is known of the anion composition except that chloride is practically if not completely absent. Thus sodium and chloride ions are confined almost entirely to the blood, whereas more than 90% of the potassium of the larva is located in the cells.

Table 2. *Inorganic composition of the tissues*

Inorganic ion	Mean concentration mM./kg. tissue water	S.D. (±mM./l.)	No. of readings
Potassium	135	28	8
Sodium	1	8	5
Calcium	7	3	4
Magnesium	35	7	4

COMPOSITION OF THE EXCRETORY FLUID

The excretory fluid of *Sialis* larvae, which is presumably produced by the Malpighian tubules, collects in the rectum which gradually distends. When it is fully distended the fluid is discharged through the anus. In order to collect this fluid larvae are narcotized with CO_2, carefully dried with filter-paper and placed on a waxed slide under a binocular dissecting microscope. The end of a capillary pipette, holding about 5 μl., is brought in contact with the opening of the rectum. This opening is normally kept closed by a sphincter muscle, but if the rectum is full of fluid, the touch of the pipette tip on the anus will cause this muscle to relax and the contents of the rectum are discharged. As the fluid is discharged it is sucked up into the pipette and in this way 2–3 μl. of fluid can usually be collected. Since the water intake of a larva weighing 100 mg. is known to be approximately 4 mg. per day at 20° C., it is probably that the excretory fluid is discharged about once a day.

If the rectum is not full then no discharge takes place but fluid can still be collected. If a slight pressure is exerted on the abdomen by the finger, the micro-pipette can be inserted through the rectal sphincter and the contents of the rectum taken up into the capillary.

It is important to be certain that the fluid collected in this way has been derived only from the rectum and not from the rest of the gut. The mid-gut opens directly into the rectum, the opening being normally closed by a sphincter just anterior to the point where the Malpighian tubules enter and if this sphincter opened mixing of the contents might occur. However, the fluid normally present in the fore- and mid-gut is a very dark brown in colour, whereas the rectal fluid is quite colourless. If any mixing occurs the latter fluid becomes coloured so any samples of the fluid which were coloured were discarded.

The results of the analysis of the fluid derived from normal larvae are shown in Table 3. Although no measurements were made of total osmotic pressure, the conductivity determinations show that the fluid is not a dilute one, as in so many fresh-water animals, but its conductivity is as much as 65% of that of the blood. On the anion side this is made up almost entirely of bicarbonate, and a large number of measurements of chloride failed to reveal the presence of this ion at all. Of the cations, sodium and potassium only account for a small percentage of this fraction. Dr B. W. Staddon, working in this Department, measured the ammonium ion concentration, and the author is indebted to him for the figure for the concentration

of this ion (100 mM./l.) which is given in the table. This concentration is sufficient to account for the rest of the cation fraction as required by the conductivity measurements.

Table 3. *The composition of the excretory fluid*

Measurement	Mean concn. (mM./l.)	S.D. (±mM./l.)	No. of readings
Conductivity as equiv. NaCl soln.	109	25	10
Potassium	4	1	6
Sodium	12	3	6
Ammonium	100	—	—
Chloride	0	—	20
Bicarbonate	91	23	7

The excretory fluid, therefore, consists largely of ammonium bicarbonate in sufficient concentration to make the total osmotic pressure of the fluid at least two-thirds that of the blood. This is markedly different from measurements on the mosquito larva, *Aedes aegypti*, made by Ramsay (1950), who showed that in this species the rectal fluid of larvae kept in distilled water was very dilute (average osmotic pressure equivalent to 12 mM./l. NaCl).

The concentration of sodium in the fluid (12 mM./l.) may be too high. It was found (Shaw, 1955*b*) that sodium was only lost slowly from the larvae when they were washed in running tap water for 3 weeks, and it seemed likely that this sodium was lost through the cuticle, together with chloride. If this is true then sodium could not be lost in the excretory fluid as well, and it is possible that the sodium found in the rectal fluid was in the process of being reabsorbed and that this was not complete when the fluid was removed.

FORMATION OF THE EXCRETORY FLUID

In view of the fact that the excretory fluid is completely different in composition from the blood, it is of great interest to know how this fluid is formed and the part the rectal epithelium plays in its formation. In order to study the equilibrium conditions which exist across the rectal wall, measurements have been made of the potential difference across this membrane. For this purpose a larva was narcotized and the abdomen dissected open and pinned out in a shallow depression in a wax block in such a way as to expose the rectum. The electrodes consisted of glass capillaries which tapered to a fine point and were filled with 1% NaCl solution. Fine silver wires coated with silver chloride were inserted through the wider ends of the capillaries. The two electrodes were held in micro-manipulators and one of them was moved into position so that its tip was beneath the surface of the blood; the other was first held in a similar position so that the out-of-balance potential between the two electrodes was measured. The second electrode was then inserted through the rectal epithelium to make contact with the rectal fluid and a measurement of the potential difference between the two electrodes made. The results of these measurements are shown in Table 4. The mean value was 24 mV. but two of

the readings (40 mV.) are much higher than the others. These two measurements were made on the rectum when it was contracted, whereas the others were taken on the fully distended rectum. The rectal wall is considerably stretched in the distended rectum, and it is possible that leakage around the electrode in these cases might reduce the measured potentials and that the higher values found in the contracted rectum may be nearer the true ones.

Table 4. *Potential difference measurements across the rectal wall*

Readings (mV.)	Mean (mV.)	Sign
20, 26, 18, 8, 26, 20, 14, 40, 26, 26, 25, 28, 40, 20	24	In all cases the blood negative to the rectal fluid

From the knowledge of the potential difference across a membrane and the concentration of an ion on one side, the concentration of this ion on the other side which would be in equilibrium can be calculated. Thus if C_r is the concentration of an ion in the rectal fluid and C_b the concentration in the blood then

$$\text{for an anion} \quad C_r = C_b \exp\left[\frac{zF}{RT}(E_o - E_i)\right],$$

$$\text{for a cation} \quad C_r = C_b \exp\left[\frac{zF}{RT}(E_o - E_i)\right]^{-1},$$

z = valency of ion, F = the Faraday, T = abs. temp., $E_o - E_i$ = the potential difference.

For a potential difference of 24 mV. the value of the exponential expression is 2·59 and the reciprocal is 0·38 at 17° C., and for the maximum recorded potential the exponential is 4·93 and the reciprocal 0·20.

Considering first the bicarbonate ion, the mean concentration in the blood is 15 mM./l. and for a potential of 24 mV. the equilibrium concentration in the rectum is $15 \times 2{\cdot}59 = 39$ mM./l. The maximum equilibrium concentration is 74 mM./l. This is less than the measured concentration of 91 mM./l. and thus it would appear that this ion is not in complete equilibrium with the blood bicarbonate. It is noticed, however, that the potential need only be 5 mV. greater than the maximum recorded in order that the electrochemical potential of this ion should be the same on either side of the membrane. Since the accuracy of the potential measurements cannot be regarded as very great, not much significance can be attached to the apparent discrepancy. The concentration of ammonium ions in the blood is very low, and the potential would require the rectal fluid ammonium ion concentration to be even lower. Since it is, in fact, very high it is clear that this ion is not distributed in the expected manner and the rectal epithelium must be either impermeable to this ion or transport it actively against the electrochemical gradient.

The concentration of sodium ions in the excretory fluid (12 mM./l.) is lower than would be expected, since for a potential of 24 mV. and a blood concentration of 109 mM./l., the equilibrium concentration would be 41 mM./l. and for the maximum

recorded potential it would be 22 mM./l. As with the bicarbonate ion the discrepancy is not a large one, and may indicate that equilibrium had not been quite reached at the time the measurements were made.

Estimations of the chloride concentration of the excretory fluid, as described above, showed that this ion is not present. Since the mean blood concentration is 31 mM./l. the minimum equilibrium concentration would be 81 mM./l. and therefore, as in the case of the ammonium ion, the membrane must be either actively or passively impermeable to this ion.

The concentrations of bicarbonate ions and sodium ions on either side of the rectal wall suggests a kind of Donnan distribution with the non-penetrating ions being ammonium on one side of the membrane and chloride and other blood anions on the other side. If this was the case then it would be expected that

$$\frac{[HCO_3']_r}{[HCO_3']_b} = \frac{[Na^+]_b}{[Na^+]_r} = \exp\left[\frac{zF}{RT}(E_o - E_i)\right].$$

The ratio of the mean bicarbonate ion concentrations is 6 and that of sodium ions 9, so that, remembering the large standard deviations of the means, the expression is approximately true. However, the possibility cannot be excluded that sodium ions are actively absorbed, at least to some extent, in the rectum.

These considerations give no information on the nature of the fluid produced by the Malpighian tubules or to what extent this is modified by the action of the rectal epithelium. It has been shown by Boné & Koch (1942) that chloride is contained in the secretions of the Malpighian tubules of the aquatic larvae of *Limnophilus* and *Chironomus*, and that this is largely removed in the rectum. Similarly, the recent measurements by Ramsay (1953*a*) on the Malpighian tubule fluid of the mosquito larva has shown that both sodium and, particularly, potassium are present and that these ions are again largely reabsorbed in the rectum.

In the case of *Sialis* larvae no attempt has been made to analyse the Malpighian tubule fluid directly, but some measurements have been made on the rectal fluid in the early stages of its formation, that is, soon after the distended rectum has been discharged, on the assumption that this fluid would resemble to some extent the fluid produced by the Malpighian tubules.

Measurements of the chloride concentration made in this way again failed to reveal the presence of chloride in this fluid. It is therefore assumed that in this larva that, unless chloride is reabsorbed very rapidly by the rectum, the Malpighian tubule fluid does not contain chloride. This seems a real difference from the findings of Boné & Koch since their technique also involved a collection of fluid from the anterior of the rectum. Similar measurements of the potassium concentration of this newly formed fluid, however, showed that this always contained a higher concentration than the fully formed fluid. Thus concentrations of 19, 36 and 26 mM./l. were obtained compared with the mean value of 4 mM./l. for the discharged fluid. Thus potassium is clearly reabsorbed in the rectum and, in this respect resembles the mosquito larva. The p.d. across the rectal wall is such that there is no necessity to postulate any active process being involved in this reabsorption.

If it is assumed that the fluid produced by the Malpighian tubules is isotonic with the blood as has been shown for the mosquito larva by Ramsay (1950, 1951), the simplest hypothesis for the composition of the Malpighian tubule fluid is that it consists of ammonium ions and sodium ions in the concentrations found in the rectal fluid and the rest of the cation fraction made up of potassium ions. This would require a potassium concentration of about 58 mM./l., a value which is not inconsistent with the concentrations of potassium found in the newly formed rectal fluid. There is no reason to suppose that the cations in the Malpighian tubule fluid are balanced by any other anion than bicarbonate, and therefore the fluid would be modified in the rectum by the reabsorption of potassium bicarbonate, a process which could take place by passive diffusion.

REGULATION OF THE BLOOD CHLORIDE CONCENTRATION

To study the regulation of the blood chloride concentration larvae were kept singly in specimen tubes containing 10 ml. of a sodium chloride solution. The solution was changed each week and the larvae were kept without food. Under these conditions the larvae would live for a very long time (2 months or more) in saline solutions not exceeding a concentration of 1% NaCl. Several series of experiments were made and for each series a different concentration of NaCl was used and this varied between 34 and 171 mM./l. Estimations of the blood chloride concentration were made on one of the larvae every 2 days and measurements were carried on for a period of 4–5 weeks. The blood chloride was found to rise gradually and then to level off when a certain equilibrium concentration had been reached. The time taken for this concentration to be reached was usually between 3 and 4 weeks. In Fig. 1 the equilibrium values for the blood chloride concentration are plotted against the value of the outside medium concentration. When the outside concentration is greater than 130 mM./l. then blood chloride tends to attain the same value, but in solutions of a lower concentration than this the internal chloride level gradually increases above that of the outside and eventually reaches equilibrium at a concentration which may be considerably greater than that outside. Thus with an outside concentration of 34 mM./l. NaCl the equilibrium value for the blood chloride is 80 mM./l. or 2·4 times greater than the outside concentration.

This is strikingly different from the behaviour of the aquatic larva of *Aedes* which was studied by Wigglesworth (1938) where the blood chloride concentration is maintained almost constant in sodium chloride solutions up to about 0·75% NaCl when the regulation begins to break down. At this concentration the external medium is almost isotonic with the blood.

Since in *Sialis* the blood chloride concentration rises above that of the external medium in the lower concentrations, some mechanism other than diffusion through the cuticle must be controlling the rate of chloride intake. Beadle & Shaw (1950), however, showed that *Sialis* larvae possessed no structures equivalent in function to the anal gills of the mosquito larvae for the uptake of ions from dilute solutions, so that the intake of chloride must, presumably occur through the gut.

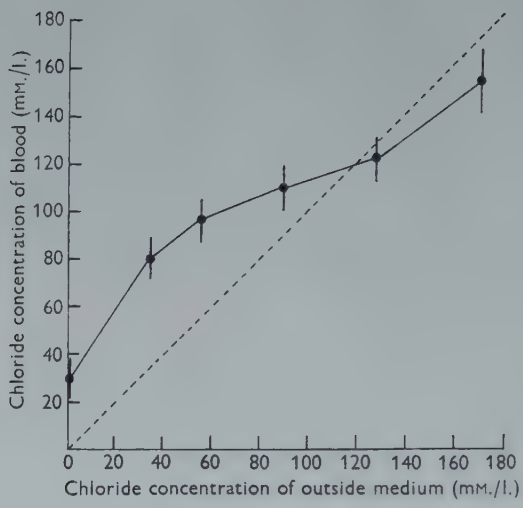

Fig. 1. The relation between the chloride concentration of the blood and the chloride concentration of the outside medium at equilibrium. The points represent mean values and the vertical lines the standard deviations of six readings.

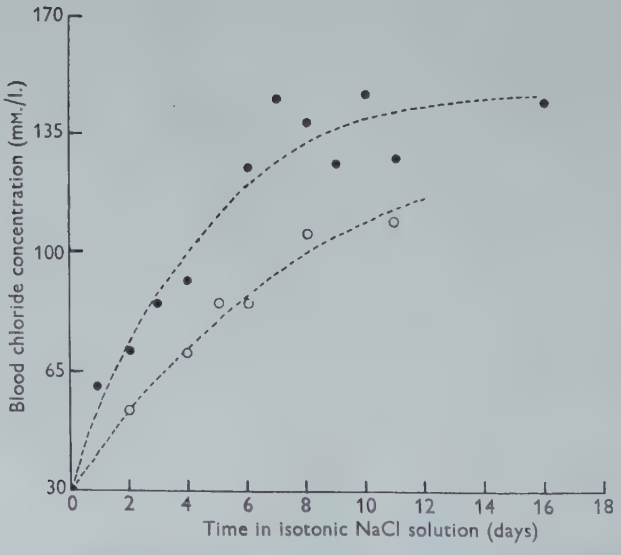

Fig. 2. The blood chloride concentration of larvae kept in an isotonic NaCl solution. ●, normal larvae; ○, larvae with necks ligatured.

This was tested by a similar series of experiments to those above, except that half of the larvae had their necks ligatured to prevent drinking. The rate of rise of blood chloride in the normal and the ligatured larvae was compared. Fig. 2 shows the results of one such series of experiments where the larvae were kept in 171 mM./l. NaCl solutions at 23° C. At this concentration the output of excretory fluid will be very small (see below) and the results will be little influenced by this. The rate of increase of the blood chloride concentration in the normal larvae is more than twice that in the ligatured larvae and equilibrium is attained in a much shorter time.

Uptake of chloride from the gut

The uptake of chloride from the gut was studied under conditions such as made it possible to obtain quantitative data on the amount taken up per day and to relate this to the concentration of solution from which it was absorbed. Larvae were first weighed and then as much blood as possible was removed from a wound in the thorax, the chloride concentration of this blood being measured. The wounds were sealed with a small blob of 'Sira' wax, each animal was weighed again and transferred to an isotonic solution of mannitol, containing a known concentration of

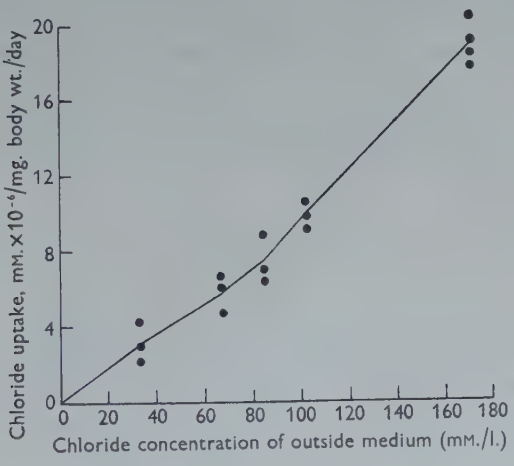

Fig. 3. Chloride uptake by the gut from solutions containing different concentrations of NaCl.

sodium chloride. With the blood volume reduced in this way, the larvae would take fluid into the gut at once. They remained in these solutions for 3 days and then were weighed again, the blood chloride concentration being once more measured. From these measurements and using an estimate for the blood weight of 56% of the body weight, the total amount of chloride taken up during the 3 days could be calculated.

The results of these experiments are shown in Figure 3, the uptake being expressed as mM. $\times 10^{-6}$ Cl/mg. body weight/day and related to the concentration of NaCl in the external solution. The amount of chloride taken up is roughly proportional to the concentration of chloride outside. In the 171 mM./l. NaCl solution, chloride will also be entering by diffusion through the cuticle, but a correction can be made

for this from a knowledge of the permeability constant of the cuticle (Shaw, 1955*b*), and if this is done the uptake is reduced to about 16 mM. $\times 10^{-6}$. This makes the proportionality better still and also, incidentally, indicates that the gut wall is a good deal more permeable to chloride than the cuticle—a conclusion that was reached also for the permeability to water (Shaw, 1955*b*).

Since chloride is taken up from solutions having the same chloride concentration as the blood, measurements of the potential difference across the mid-gut wall were made to see if an active process was involved in this transfer. The technique was the same as used for measurements on the rectum, and the electrode was either pushed through the wall of the mid-gut or passed up from the rectum, through the sphincter, into the mid-gut. The results are shown in Table 5. The mean value is 18 mV. and the blood is positive to the mid-gut fluid, whereas it was negative to the rectal fluid. The equilibrium chloride concentration in the mid-gut is therefore lower than the concentration in the blood and, as before, is given by

$$C_{\text{mid-gut}} = C_b \exp\left[\frac{zF}{RT}(E_o - E_i)\right]^{-1} = 15{\cdot}2 \text{ mM./l.}$$

Thus the measured uptake of chloride can be explained by the passive diffusion of chloride through the mid-gut wall. Whether chloride can be absorbed from solutions less concentrated than 15 mM./l. or not still requires investigation.

Table 5. *Potential difference measurements across the mid-gut wall*

Electrode position	Potential difference (mV.)	Mean (mV.)	Sign
Through mid-gut wall Through sphincter	6, 10, 20, 30, 22, 20 14, 20, 18, 20	18	Blood positive to mid-gut

Output of chloride in the excretory fluid

Although it has already been established that chloride is not present in the excretory fluid of *Sialis* larvae living in tap water, the fact that the blood chloride concentration reaches an equilibrium value when the larvae are living in sodium chloride solutions suggests that the chloride uptake from the gut must eventually be balanced by a corresponding output in the excretory fluid. In order to test this, measurements were made of the chloride concentration of the excretory fluid of these larvae. When the larvae were living in the higher concentrations of sodium chloride the volume of the excretory fluid was considerably reduced, and therefore to make measurements on the excretory fluid of these larvae they were replaced for 2 days in tap water. This stimulated the production of rectal fluid once more and then measurements were made of the chloride concentration in the rectal fluid and in the blood. Fig. 4 shows the results of these determinations. The chloride concentrations of the excretory fluid are related to the blood concentration at the time of the measurement. The rise in chloride concentration of the rectal fluid is slow at first—when the blood concentration is twice the normal value the level has only reached

about 10 mM./l. With further increases in the blood concentration the rise in rectal fluid concentration is steeper until at a blood level of about 100 mM./l. the rise becomes very steep and the concentration of chloride in the excretory fluid approaches that of the blood when the latter is about 120 mM./l. There is no evidence that the concentration ever exceeds that of the blood.

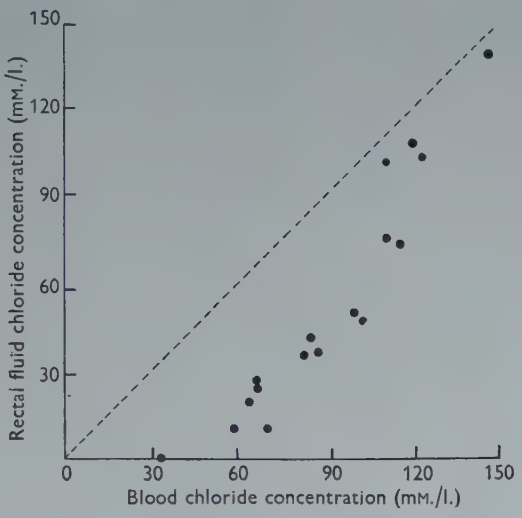

Fig. 4. Relation between the chloride concentration of the blood and the chloride concentration of the rectal fluid.

Chloride balance

At the equilibrium concentration of chloride in the blood, the output of chloride lost must be exactly balanced by the amount of chloride absorbed. Chloride will be lost in two ways: first, in the rectal fluid if the blood chloride level is above normal; and secondly, by diffusion through the cuticle if the concentration of chloride in the blood is greater than that of the external medium. Thus if no chloride was absorbed, then the rate of loss of the blood chloride $= V_r C_r + \frac{C_b - C_o}{T} W$, where V_r = volume of rectal fluid per day, C_r = Cl concn. of rectal fluid (function of C_b); C_b = Cl concn. of blood and C_o = Cl concn. of external medium. T = time constant for diffusion of Cl through cuticle and is 20 days at 14° C. W = blood weight.

This value for the loss of chloride can be computed for the equilibrium concentrations given in Fig. 3 and C_r calculated from Fig. 6. $V_r = 4\%$ of the body weight per day at 20° C. for larvae in tap water (Shaw, 1955*b*). Making this calculation for external concentrations of 34, 58 and 85 mM./l. gives a rate of chloride loss as 2·64, 2·61 and 2·55 mM $\times 10^{-6}$/mg. body weight/day, and at equilibrium this must be balanced by an equal chloride uptake. Now comparing these rates of chloride uptake with the values obtained under conditions where drinking had been induced experimentally (Fig. 5), they indicate, particularly in the higher concentrations, that

a considerably smaller amount of chloride is absorbed per day. This suggests that the amount of drinking is controlled by the larvae so that the rate of chloride absorption is kept at a fairly constant level.

REGULATION OF THE BLOOD SODIUM CONCENTRATION

The regulation of the blood sodium concentration was studied in experiments similar to those described for chloride. Larvae were kept in sodium chloride solutions and measurements made of the blood sodium concentration until the equilibrium concentration had been reached. The results of these experiments are shown in Fig. 5. In contrast to the large increases in blood chloride which occurred in the lower concentrations of the external medium, the blood sodium concentration only

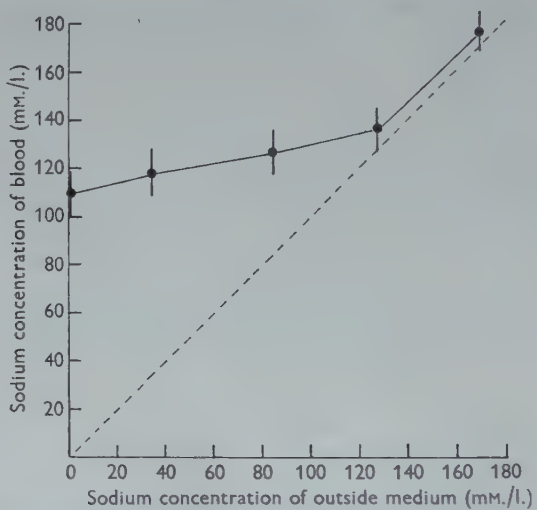

Fig. 5. The relation between the sodium concentration of the blood and the sodium concentration of the outside medium at equilibrium. The points represent mean values and the vertical lines are the standard deviations for six readings.

increases slightly in solutions of sodium chloride up to about 130 mM./l. when the two concentrations become approximately the same. At higher concentrations of the external medium the rise in the blood sodium concentration is steeper and tends to follow that of the outside solution.

Despite the difference in regulation of the chloride ion as between *Sialis* larvae and mosquito larvae, the behaviour of the sodium ion appears to be similar. Ramsay (1953*a*) gives the mean sodium concentration of the blood of mosquito larvae in distilled water as 87 mM./l. and this rises only to 100 mM./l. when the larvae are placed in 85 mM./l. NaCl, whereas in 171 mM./l. solutions (Ramsay, 1951) the concentration rises to 208 mM./l.

In *Sialis* larvae, owing to the different powers of regulation of sodium and chloride ion concentrations in the blood, the ratio of sodium to chloride ions in the blood decreases markedly when the larvae are kept in saline solutions. This increase in the

chloride concentration must, therefore, occur at the expense of the rest of the blood anion fraction, which as has been seen is probably made up by organic acids. Whether these substances are removed from the blood by excretion or by metabolism or whether they are temporarily stored in the tissues is not known.

The differences in regulatory powers of the larvae with respect to sodium and chloride must be due either to different rates of uptake of these ions from the gut or a differential output in the excretory fluid, or to a combination of both these processes. Both the absorption of sodium and its loss in the rectal fluid have been studied in the same way as for chloride.

Uptake of sodium from the gut

The fact that the equilibrium concentrations of blood sodium, shown in Fig. 5, are higher than the sodium concentrations of the external media, suggests that sodium is being absorbed from the gut in the same way as chloride. Experiments were carried out to demonstrate this and to compare the rates of uptake of sodium and chloride. Blood samples were removed from weighed larvae and the sodium and chloride concentrations measured. The larvae were placed in 85 mM./l. NaCl or in tap water as a control after being weighed again. The larvae remained in these media from 2 to 4 days, then they were weighed a third time and the sodium and chloride concentrations measured once more. The control experiments served as a further check on the estimate of the average blood volume and to demonstrate, formally, that sodium as well as chloride was not absorbed from dilute solutions. The results of these experiments are shown in Table 6. Minus signs before the figures for uptake in tap water indicate that there has been an apparent loss. This measurement serves as a control of the accuracy since there should be neither uptake or loss in these cases and thus a difference of 1–2 mμM./mg./day cannot be regarded as significant. The mean values for the uptake of chloride (10·1) and sodium (9·6) from the 85 mM./l. solution indicate that these two ions are taken up together, in approximately equal amounts, through the gut. The individual measurements, also, although different in some cases, are not sufficiently different to be significant in view of the variable results in tap water. Now the potential difference measurements shown in Table 5 indicated a mean value of 18 mV. across the mid-gut wall

Table 6. *The uptake of sodium and chloride from the gut*

(mM. × 10^{-6}/mg. body weight/day.)

Larvae in tap water			Larvae in 85 mM./l. NaCl		
Specimen no.	Cl	Na	Specimen no.	Cl	Na
1	−0·7	0·0	5	8·8	5·5
2	−3·0	−2·3	6	6·9	8·0
3	−1·6	1·6	7	6·4	8·1
4	−1·8	0·0	8	12·3	12·6
Mean	−1·7	−0·2	9	7·8	6·0
			10	18·2	17·1
			Mean	10·1	9·6

and the equilibrium concentration of sodium ions in the gut would be 218 mM./l. Since uptake occurs from a 85 mM./l. solution, this transport of sodium ions must involve an active process, presumably occurring in the mid-gut wall. The chloride ions which are absorbed at the same time and in the same amounts may be penetrating by passive diffusion through the mid-gut wall.

The sodium concentration of the excretory fluid

Since sodium and chloride ions appear to be absorbed from the gut in equal amounts, the differences which were found in the equilibrium concentrations of these ions in the blood could be due to different rates of excretion of these ions. To test this measurements were made of the sodium concentration of the excretory fluid in the same way as for the chloride. Fig. 6 shows the values obtained for the sodium concentration of this fluid related to the concentration of sodium in the blood measured at the same time. At the normal blood sodium level

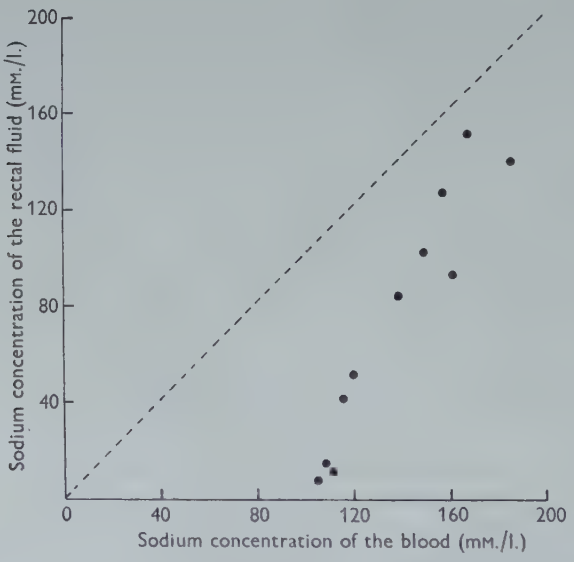

Fig. 6. The relation between the sodium concentration of the blood and the sodium concentration of the excretory fluid.

the concentration of sodium in the rectal fluid is low, but as the blood concentration increases the amount of sodium excreted rises rapidly so that when the blood contains about 170 mM./l. of sodium the rectal fluid concentration is about the same. The sodium concentration of the rectal fluid is approximately proportional to the amount by which the blood sodium concentration exceeds its normal value, and in this respect contrasts with the chloride concentration of the rectal fluid which follows a curve of progressively increasing steepness with increasing blood concentration.

This differential effect in the excretion of these two ions can account for the decrease in the Na/Cl ratio which occurs when larvae are kept in sodium chloride

solutions. By comparing Figs. 4 and 6 it will be seen that at the lower blood concentrations of sodium and chloride, the concentrations of these ions in the rectal fluid will be quite different but as the blood concentrations are increased so the rectal fluid concentrations become closer together. This is illustrated by the only three cases in which both sodium and chloride were determined on the same sample of rectal fluid. The concentrations of Na/Cl were 103/48, 90/51 and 140/113 mM./l. respectively.

REGULATION OF THE BLOOD POTASSIUM CONCENTRATION

The normal concentration of potassium in the blood has been shown to be quite low (mean = 5 mM./l.) and the mechanisms by which this low level is maintained have been studied. First, the effect on the blood potassium concentration of keeping the larvae in solutions of potassium chloride was measured. The technique was the same as for the measurements on sodium and chloride and the results for the exposure of the larvae to solutions of 34 and 171 mM./l. solutions of KCl are shown in Fig. 7, where the blood potassium concentration is related to the number of days in the KCl solution.

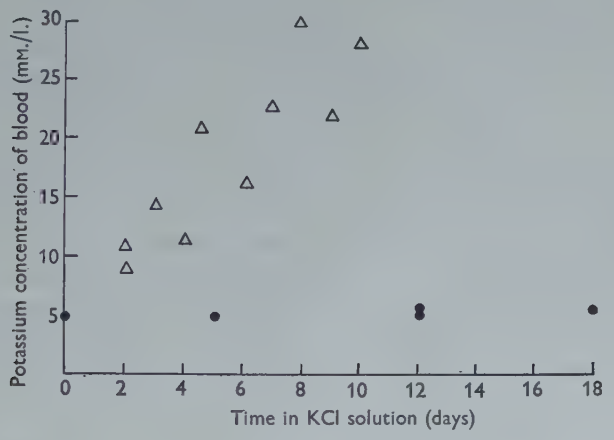

Fig. 7. The increase in the concentration of potassium of the blood of larvae kept in KCl solutions. △, larvae in 171 mM./l. KCl; ●, larvae in 34 mM./l. KCl.

In outside solutions of concentrations up to 34 mM./l. KCl larvae would live for a very long time and the measurements of the blood concentration showed that the potassium level was not increased. If the concentration of the outside medium was increased then the blood potassium concentration rose and the larvae did not live for very long. For this reason it was not possible to determine equilibrium concentrations in the same way as for sodium and chloride. In 85 mM./l. KCl solutions the larvae would live for about 2 weeks, and if the concentration was increased to 171 mM./l. KCl they would survive for little more than a week. This is in marked contrast to the behaviour of the larvae in NaCl solutions where they would live for an almost indefinite period in isotonic solutions.

The effect of increased blood potassium on the larvae

Before the larvae kept in isotonic solutions of potassium chloride finally die they pass through a number of fairly well-defined states in which they show changes in their normal behaviour and these symptoms can be correlated with the concentration of potassium in the blood. Normal larvae, kept in the laboratory, are quite active—they will immediately right themselves by strenuous muscular activity if they are turned over, and if they are touched on the head they give a characteristic response whereby the whole body is jerked quickly backwards.

Major differences in behaviour show themselves when the blood potassium concentration reaches about 10 mM./l. The larvae are much more sluggish than normal and will not give the characteristic backing-away response so readily. Between blood concentrations of 10–15 mM./l. the larvae have difficulty in righting themselves if they are turned over, although they can maintain their normal balance if left alone. At concentrations between 15 and 20 mM./l. the larvae are usually lying on their backs, but they are just able to right themselves if they are stimulated. Above 20 mM./l. they are always on their backs and quite unable to maintain their correct position. They are quite still, although stimulation will still initiate certain reflexes, such as the bending of the legs. Any further increase in the potassium content results in the death of the larva. These physiological effects are to some extent reversible; if the blood concentration has not risen above about 15 mM./l. a return of the larva to tap water and subsequent reduction of the blood potassium level appears to restore it to normal, but if the rise has been greater than this the larvae may recover some of their former activity, although the restoration is not complete and some permanent damage has clearly been done. In as much as the physiological effects of increased blood potassium first appear as modifications of normal behaviour patterns and muscular action remains intact, it would seem that the initial effects are on the central nervous system rather than the muscular system. If this is so, then this insect, unlike the locust (Hoyle, 1953) cannot have the whole of its nervous system surrounded by a sheath acting as an effective barrier to the diffusion of potassium ions.

Uptake of potassium from the gut

The uptake of potassium and chloride from the gut was studied by means of experiments similar to those described for sodium and chloride. A known amount of blood was removed from a weighed larva and the potassium and chloride concentrations measured. The larva was replaced in a solution of 34 mM./l. KCl weighed again after 2 days and the blood concentrations measured once more. Calculations of the uptake of chloride under these conditions, in four experiments, gave the following values: 3·7, 3·8, 3·2 and 5·4 mμM./mg. body weight/day. These values are very similar to those obtained for the uptake of chloride from 34 mM./l. NaCl solutions. Thus the chloride uptake seems to be the same from both the chloride solutions and again there is no evidence of any active process being involved.

Similar calculations, however, for the simultaneous uptake of potassium by the gut give a considerably lower value—0·8, mean of three readings compared with the mean of 4 mμM./mg. body weight/day for the chloride. The production of excretory fluid, however, continues during this absorption and this fluid, as will be shown, may contain large amounts of potassium, so the value deduced for the uptake actually represents the difference between the uptake and the loss in the excretory fluid. This loss, however, is not likely to be greater than 1·6 mμM./mg body weight/day so that it is possible that the true uptake of potassium is still considerably less than that of the chloride. Further experiments would be required in order to establish this firmly. The concentration of the outside medium in these experiments is much greater than that of the blood (34 mM./l. against 5 mM./l.) and therefore, despite the direction of the potential difference across the gut wall, the uptake could be due to passive diffusion. Again, more experiments are required before the presence of an active process can be altogether excluded, but it seems clear that if a transporting mechanism is present for potassium, it does not absorb this ion as fast as does the corresponding mechanism for sodium ions.

Potassium concentration of the excretory fluid

In solutions of KCl, potassium ions will be entering the larvae through the cuticle and through the gut. The facts that the blood potassium concentration does not rise in an outside concentration of 34 mM./l. KCl and that in an isotonic solution of KCl it increases only slowly compared with the increase in chloride concentration suggest that large amounts of potassium can be eliminated in the excretory fluid. This was demonstrated by measurements of the potassium concentration of the excretory fluid made on larvae living in solutions of KCl. Some of these larvae were normal, others had their mouths waxed over in order to prevent any contamination of the excretory fluid with potassium chloride solution taken in through the mouth. The results of these experiments are shown in Fig. 8 where the potassium concentration of the excretory fluid is related to the concentration of the blood measured at the same time. The rise in the concentration of potassium is very rapid—small increases in the blood concentration very quickly raise the concentration of the rectal fluid above that of the blood and rise continues, with increasing blood concentration, until a value of about 100 mM./l. is reached. This is very different from the results obtained for measurements of sodium and chloride where the concentrations of these ions in the rectal fluid never increased beyond that in the blood. When the concentration of potassium in the rectal fluid reaches about 100 mM./l. it seems to level off, but more determinations are required on larvae with high blood potassium concentrations in order to establish this.

Similar high concentrations of potassium have been found by Ramsay (1953*a*) in the rectal fluid of mosquito larvae kept in 85 mM./l. KCl for 3 weeks—the mean concentration was 90 mM./l.

Conductivity measurements were also made on the excretory fluid produced by *Sialis* larvae living in isotonic KCl solutions. The results are shown in Fig. 9 where

the conductivity of the rectal fluid is related to the number of days that the larvae has spent in the isotonic KCl solution. The conductivity, like the potassium concentration also rises steeply and then levels off after about 4 days at a value corresponding to a 200 mM./l. NaCl solution. Now this value is probably not much greater than the conductivity of the blood at the same time, since the normal blood

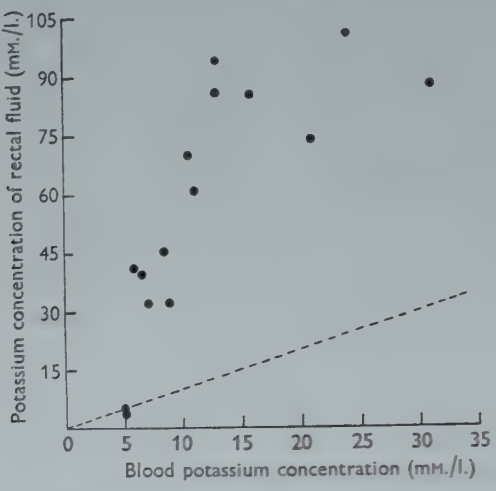

Fig. 8. The relation between the potassium concentration of the blood and the potassium concentration of the rectal fluid.

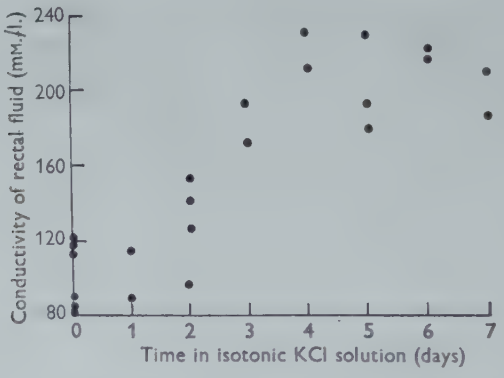

Fig. 9. The increase in conductivity of the rectal fluid of larvae kept in isotonic KCl solutions.

conductivity will have been increased by KCl which has diffused into the blood during the 4 days. Thus the concentration of the rectal fluid increases until it is approximately isotonic with the blood and then no further increase occurs. By reference to Fig. 7 it will be seen that in 4 days the blood potassium concentration will have risen to 15 mM./l. at which level the maximum concentration of potassium in the rectal fluid is reached (Fig. 8). Thus the maximum concentration of the excretory fluid is attained when the potassium concentration also reaches its maximum.

The value for the conductivity, however, is much greater than that due to the potassium alone and the differences could be accounted for approximately if ammonium bicarbonate is present also in the rectal fluid in its normal concentration. If this is so then this very efficient potassium regulating mechanism may be limited by the fact that the rectal fluid cannot become hypertonic to the blood and still excretes the normal concentration of ammonium bicarbonate, even under conditions of extensive potassium excretion.

WATER BALANCE

Just as an equilibrium concentration of any inorganic ion in the larva is established when the amount of that ion absorbed is balanced by that which is excreted, so the water content of the larva will be in equilibrium when the rate of water intake is equal to the rate of elimination of the excretory fluid.

In larvae kept without food daily weight records kept for a period of 3 weeks showed little change. Measurements of water permeability (Shaw, 1955*b*) indicated that drinking of water did not occur in these circumstances so that the volume of excretory fluid produced must be equal to the volume of water which enters the larva through the cuticle.

Drinking, however, does occur if the blood volume is reduced or if salt is present in the outside medium. A combination of these two conditions has been used as a method for studying the uptake of water from the gut and the relation of this to the simultaneous absorption of salts.

It has already been shown (Shaw, 1955*b*) that if water is taken into the gut it is absorbed osmotically and that the gut wall is much more permeable to water than the cuticle. No water is taken up from solutions of mannitol isotonic with the blood even if these are swallowed. In the following experiments the effect of drinking isotonic solutions of salts was investigated. The technique was similar to that used for the study of the uptake of salts from the gut: blood was removed from weighed larvae, they were reweighed, placed in one of the isotonic salt solutions and finally a daily weight record kept for a period of 5–6 days. The results of the experiments are shown in Fig. 10. Six larvae were used for each experiment but the results for only one or two are given in order not to confuse the figure. There was, however, little variation and the selected examples are typical of the behaviour of the whole group. Where necessary controls were also carried out by repeating the same experiment on a group of larvae with their necks ligatured to prevent drinking. Isotonic solutions of NaCl, KCl, $CaCl_2$ and $NaHCO_3$ were used for these experiments. In the case of the NaCl solutions, the weight of the larvae gradually increased, whereas that of the ligatured larvae did not. The increase was continued until the original blood volume had been restored and in a few cases the original weight was exceeded. The larvae were perfectly normal in behaviour during this process of blood regeneration and would continue to live for long periods afterwards. The average rate of water absorption was about 5% of the body weight per day, and therefore was actually greater than the normal rate of water uptake through the cuticle (4% body weight/day at 20° C.; Shaw, 1955*b*).

In isotonic $CaCl_2$ solutions no uptake of water occurs and in this respect they resemble the isotonic mannitol solution; in the isotonic KCl solution the weights of the larvae actually decrease. In the $NaHCO_3$ solution, water uptake does take place at first, but the larvae do not live long in this solution. The rate of uptake is approximately the same as in the NaCl solution.

Now it is clear that this absorption of water cannot be due to osmotic forces since the solutions are isotonic with the blood and this is confirmed by the fact that water is not absorbed from the mannitol solution. Since the uptake occurs from some salt solutions, it suggests that this water transport is associated with the simultaneous absorption of salts which is occurring. It cannot be associated with the uptake of chloride since this is certainly occurring in the KCl solutions. Active transport of

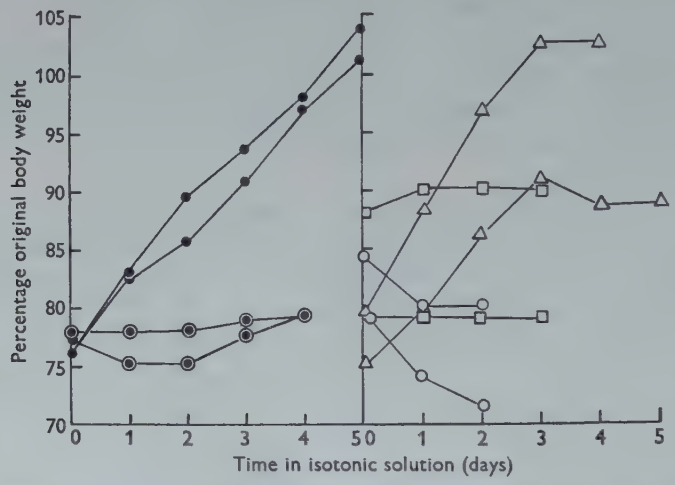

Fig. 10. The recovery of body weight of larvae with reduced blood volume placed in isotonic solutions. ●, larvae in NaCl; ⊙, the same but with necks ligatured; △, in $NaHCO_3$; □, in $CaCl_2$; ○, in KCl.

sodium ions has been shown to be taking place in NaCl solutions, and since the water uptake occurs also in this solution and in the $NaHCO_3$ solution, the simplest explanation of these facts is that this water transport is in some way linked with the active uptake of sodium by the gut wall.

Since chloride is absorbed at the same rate as sodium from NaCl solutions, and water is absorbed at the same time, then the total absorption can be regarded as that of a solution of NaCl in water. The concentration of chloride in this solution was measured by repeating the above experiments on the regeneration of blood from an isotonic NaCl solution, but at the same time measuring the blood chloride concentration before and after the experiment and hence calculating the chloride uptake. The experiments were repeated using solutions containing different concentrations of NaCl but maintained isotonic with the blood by the addition of mannitol. By measuring the water uptake and the simultaneous absorption of chloride the concentration of chloride in the absorbed water was calculated and the results are shown

in Table 7. The concentration of chloride in the water in all the experiments is roughly constant and bears no relation to the concentration of NaCl in the medium from which it was absorbed. The mean value for the seventeen experiments is 155 mM./l. (S.D. ± 24 mM./l.), and thus is considerably greater than the concentration of either chloride or sodium in the blood. It is, however, nearly isotonic with the blood, and since the experiments covered a fairly wide range of concentrations of the outside medium, it seems very likely that sodium and chloride are absorbed from the gut in the form of a solution of NaCl which is approximately isotonic with the blood.

Table 7. *The concentration of chloride in the fluid absorbed by the gut from an isotonic solution containing* NaCl

Concn. of NaCl in external medium (mM./l.)	Calculated chloride concn. of fluid absorbed by gut (mM./l.)	Mean (mM./l.)
34	109, 167, 191	156
68	147, 152, 156	152
85	115, 128, 132	125
103	127, 150, 166, 188, 195	165
171	146, 178, 181	169

There are, then, three ways in which water may enter the body of the larva. First, water is taken up osmotically through the cuticle at a rate of about 4% of the body weight per day at 20° C. Secondly, water may be absorbed osmotically through the gut wall when water or dilute solutions are swallowed, and intake by this method may be large by reason of the high permeability of the gut. Finally water may be transported across the gut wall by a process which is probably linked to the active absorption of sodium ions.

Water output

The only known route for the loss of water from the larvae is by means of the excretory fluid. Since, as has been demonstrated in the preceding section, the water intake varies according to circumstances it is interesting to find to what extent the larvae are able to regulate the volume of the excretory fluid. Of the possible factors which might be involved in controlling rectal fluid volume, two have been investigated experimentally. The first was effect of the reduction of the blood volume which had been found to stimulate water uptake and the second the direct effect of altering the water intake.

In the first series of experiments, blood was removed from weighed larvae in the usual way and they were replaced in tap water, with their necks ligatured to prevent drinking which would normally occur under these circumstances. Daily weight recordings were taken and the results are shown in Fig. 11 where the weight changes expressed as percentage of the initial body weight are related to the time after they were placed in tap water. No significant weight changes occur, and this must mean

that the normal volume of excretory fluid is still being produced since the water intake through the cuticle will not be affected by the experiment. This is confirmed by the fact that when a ligature is tied round the last abdominal segment of these larvae to prevent excretion then the weight increases in an amount corresponding approximately to the expected water uptake through the cuticle. Therefore one can conclude that a reduction of the blood volume has no effect on the production of excretory fluid if the normal rate of water intake is maintained.

If the same experiment is repeated on larvae whose necks have not been ligatured then drinking occurs, and the increase in body weight is very large; the blood volume is practically restored in one day (Shaw, 1955*b*). In this case the water

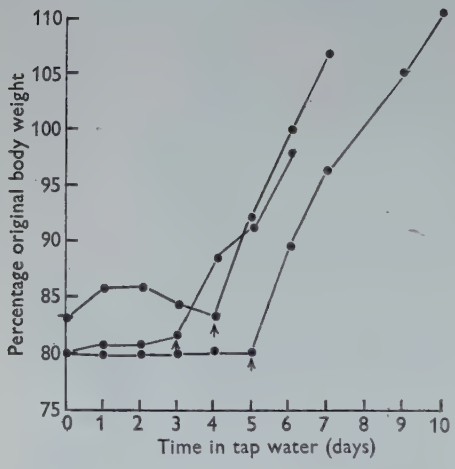

Fig. 11

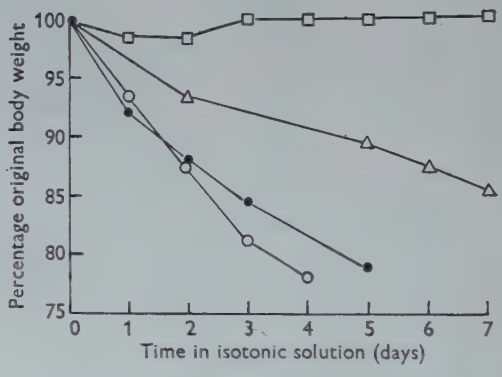

Fig. 12

Fig. 11. The recovery of weight of larvae, with necks ligatured, after removal of blood. Ligatures were also tied round the last abdominal segment at the points indicated by the arrows.

Fig. 12. The loss of weight of larvae, with necks ligatured, kept in isotonic solutions. □, larvae in isotonic NaCl; ○, in mannitol; ●, in KCl; △, in liquid paraffin.

intake is increased beyond the normal level, but this does not stimulate an equivalent output of excretory fluid. It should be noted, however, that this increased uptake of water need not necessarily lead to a dilution of the blood because of the operation of the compensatory mechanism (Beadle & Shaw, 1950), whereby the blood osmotic pressure can be maintained, probably by the addition to the blood of amino-acids.

The effect of reducing the water intake on the production of excretory fluid was studied. Osmotic uptake of water was prevented by putting larvae into solutions isotonic with the blood and weight records were taken. Some larvae were also kept in liquid paraffin in which they would live for many weeks and in this way all contact with external water was prevented. In all experiments larvae with and without neck ligatures were used, and the results obtained for the former group are shown in Fig. 12, where as before one record only is presented in each case. The record shown, however, is typical of the behaviour of each group.

The loss of weight of the larvae in isotonic KCl and mannitol occurs at a rate of about 6% of the body weight per day, although this rate tends to fall off as the blood volume is reduced and there is no doubt that, at least at the start, excretory fluid is being produced at the normal rate despite the fact that there is no water intake. In the case of the liquid paraffin the rate of weight loss is somewhat less, but further experiments would be required to establish the significance of this difference. However, in the isotonic NaCl solution the behaviour of the larvae is quite different, for in all the animals (20) no large fall in weight occurred, and in many the initial weight was maintained. In this solution, therefore, the production of excretory fluid must have practically ceased and this conclusion receives support from the fact that whereas it is easy to collect a sample of rectal fluid from larvae kept in isotonic KCl solution it is never possible to collect very much from the specimens in the NaCl solution.

In the experiments with unligatured larvae the results were approximately the same except again in the case of NaCl: here the larvae actually increased in weight

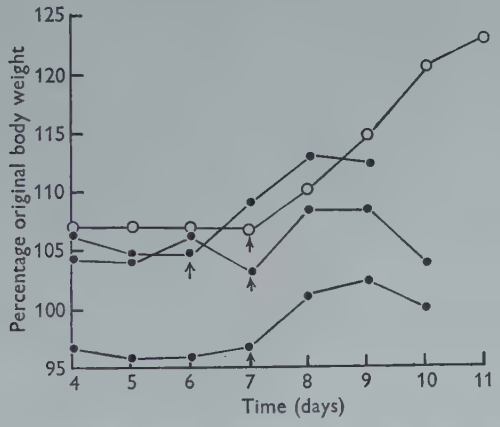

Fig. 13. Changes in weight of larvae kept in isotonic NaCl and then transferred to tap water as indicated by arrows. ●, normal larvae; ○, larvae with a ligature tied round the last abdominal segment.

in the first few days and in this case there is little doubt that intake of water through the gut wall has been taking place by means of the transport mechanism discussed above.

The effect of the NaCl solution in reducing the flow of excretory fluid is not immediately reversed when the larvae are returned to tap water. Larvae kept in isotonic NaCl reach a constant weight after a few days; if they are now transferred to tap water (Fig. 13) then the weight increases again during the first day or two, then remains constant or falls again. This weight increase must be due to the water taken up through cuticle which has not been excreted as rectal fluid.

As with uptake of water from the gut in isotonic NaCl, this reduction in excretory fluid volume appears to be due to some activity on the part of the sodium ions. The only obvious difference between the isotonic NaCl and KCl solutions is that in the former case sodium will be diffusing in to the larva. It is possible that it is the

increase in sodium ion concentration of the blood which brings about the reduction in excretory fluid production—this is supported by the fact that if the sodium concentration of the blood is raised by keeping the larvae in NaCl solutions then the flow of rectal fluid does not start again immediately after the larvae have been replaced in tap water (Fig. 13)—the blood concentration may have to be reduced again before the fluid can be produced once more.

Water output, therefore, in the form of the excretory fluid normally balances the water intake through the cuticle. The volume of this fluid is not altered by changes in the blood volume nor is it affected by a reduction in the normal intake, nor does it keep pace with large increases in the rate of water uptake. It is, however, reduced by the influx of sodium ions into the blood and may be controlled by the concentration of sodium in the blood.

Water balance in saline solutions

Sialis larvae live for long periods in NaCl solutions which are isotonic with, or hypotonic to, the blood. In more concentrated solutions than this, however, they will not live so long. For example, in solutions which are 50% greater than isotonic the larvae only live for a fortnight or so, and in solutions twice as concentrated as the blood, they die in a few days. The water relations of the larvae living in these solutions have been studied. Weight changes have been recorded for larvae, both with and without neck ligatures, placed in solutions containing 0, 85, 171, 256 and 342 mM./l. NaCl, and Table 8 gives the average daily weight changes for a period of 3 days.

Table 8. *Weight changes occurring in larvae placed in saline solutions for a period of 3 days*

Concn. of external medium (mM./l.)	Mean gain in weight per day. Percentage of initial body weight	
	Normal larvae	Larvae with necks ligatured
0	0	−1·8
85	0·1	−1·7
171	2·0	−1·5
256	−0·2	−3·5
342	−11·8	−5·3

In tap water larvae with their necks ligatured show a small loss of weight, and this rate of loss is found also in similar larvae in 85 and 171 mM./l. NaCl solutions. In contrast the normal larvae lose no weight in tap water or in 85 mM./l. NaCl, and in the isotonic solution a gain in weight is found due to the uptake of water from the gut and the reduction of the rectal fluid volume. In hypertonic solutions, the normal osmotic uptake of water is replaced by an osmotic loss. This is demonstrated in the larvae with ligatured necks in the 256 and 342 mM./l. solutions, where increased losses in weight are found. The difference between these losses and the loss

of the ligatured larvae in tap water are approximately equal to the expected loss of water by osmosis through the cuticle.

The behaviour of the normal larvae in 256 and 342 mM./l. solutions is interesting. In the former solution the larvae are just able to maintain their body weight and the uptake of water from the gut must be just sufficient to balance the loss by osmosis through the cuticle and also through the gut wall itself. The eventual death of these larvae is probably due to the rise in blood osmotic pressure caused by the penetration of salt. In the 342 mM./l. solution the loss of weight is very great and the larvae die in a few days, presumably from dehydration. The weight loss is much greater than from the ligatured larvae in the same solution, and at this concentration the mechanism of water uptake from the gut must break down so that the recorded loss is due to exosmosis through the gut, in addition to that through the cuticle.

DISCUSSION

The composition of the blood as far as the cations are concerned is not very different from that found in many fresh-water and terrestrial animals, for sodium accounts for a large proportion of the cation fraction and potassium is present only in relatively small amounts. The high concentrations of calcium and, particularly, of magnesium are the most unusual feature, although this seems to be a characteristic of insect blood. The anion composition is peculiar, however, in that chloride represents only a small fraction, and this again seems to be a common feature among insects. It would be of great interest to know what substances make up the rest of this fraction and in what way their concentration in the blood is regulated. Results already discussed suggest the presence of organic acids, which may well be dicarboxylic amino-acids. These acids have already been found in a number of terrestrial insects—for example, Auclair & Dubreuil (1953) found quantities of glutamic acid varying from 10 to 200 μg. per 100 μl. and aspartic acid from 4 to 100 μg. per 100 μl., in the blood of a variety of insects. Little is known of the organic composition of the blood of aquatic insects: Raper & Shaw (1948) described the most important amino-acids in the blood of the aquatic larva of *Aeschna cyanea*. No appreciable quantities of these dicarboxylic acids were found, but in these larvae the chloride concentration is unusually high (94 mM./l.).

The maintenance of the normal composition of the blood, at least in respect of the three ions which have been studied, is brought about by the activity of the Malpighian tubules and rectum in producing an excretory fluid of varying composition. In the formation of this fluid in unfed larvae it would appear that a system of potassium secretion by the Malpighian tubules and subsequent reabsorption in the rectum, as described by Ramsay, first for the mosquito larva (1953*a*) and then for a variety of other insects (1953*b*), operates, although the concentration of potassium in the tubules may be rather lower in *Sialis* than in the other insects. This circulation still takes place despite the fact that a concentrated excretory fluid is being produced. It is possible that some sodium is reabsorbed in the rectum, but there is no evidence that chloride is present in the fluid at all. Whether this is related to the

high concentration of bicarbonate present is not known, but it would be very interesting to have comparable data on the regulation of the chloride ion in other aquatic insects.

If the regulatory powers of *Sialis* larvae with respect to the three ions are compared, differences are noticed which may well be correlated with the physiological requirements of the larvae for these ions. Thus for the potassium ion, the excretory system is well adapted for its speedy removal from the blood if the blood concentration rises. This can be associated with the very marked detrimental effect that a small rise in the blood concentration of potassium has on the neuro-muscular system of the larvae. The removal of the ion is brought about in two ways: first, the volume of rectal fluid does not decrease with increasing concentration of potassium in the blood, despite the fact that at the same time the blood osmotic pressure may be increasing; and secondly, the concentration of the rectal fluid is rapidly increased (so that it becomes roughly isotonic with the blood) by the addition of large amounts of potassium which raise the concentration of this ion in the fluid to a level well above that of the blood.

The regulation of the chloride ion concentration of the blood, although effected by the same system, is of a different nature. Increases in the blood concentration of over 100% of the normal value only result in a small output in the excretory fluid and as a result, if chloride is present in the outside medium, chloride tends to be accumulated in the blood. Now large increases in the blood chloride level have no adverse effect on the larvae, and it would appear that, for the chloride ion, the regulatory system is adapted for the conservation of chloride. This idea also receives support from the fact that chloride appears to be completely absent from rectal fluid of the unfed larvae. This emphasis on conservation of chloride may well be associated with the absence from these larvae of any structure capable of absorbing chloride from dilute solutions. It is interesting to find that in the mosquito larva, where such structures do exist (Koch, 1938), that the regulation of the chloride concentration of the blood of larvae in saline solutions is much more rigid (Wigglesworth, 1938).

The regulation of the sodium ion concentration occupies, in some ways, an intermediate position between that of potassium and that of chloride. Regulation is effected by excretion of sodium in the rectal fluid and the concentration of sodium in this fluid rises more rapidly than does the concentration of chloride, for equivalent increases in the blood concentration, but does not increase nearly as quickly as the concentration of potassium. Now increases in the blood sodium concentration have no adverse physiological effect on the larvae but will lead to an increase in the total osmotic pressure of the blood. Larvae can only tolerate relatively small increases in this, and therefore they are unable to store sodium in the blood in the same way as chloride, although conservation of sodium is as much a problem as the retention of chloride. Sodium storage can be effected, however, by a different mechanism which makes use of the special effect of the sodium ion on the water balance of the larvae. If sodium is absorbed from the gut in the form of an isotonic solution and the influx of sodium into the blood somewhat reduces the rectal fluid volume, then this solu-

tion could be retained in the blood by an increase in the blood volume. Since the solution is isotonic, the osmotic pressure will not be raised, and since the sodium concentration of the solution is not much greater than that of the blood, the blood sodium concentration will also only be raised by a small amount. The behaviour of the excretory system in this respect, recalls a similar behaviour of the vertebrate kidney: if isotonic NaCl is injected in the blood of a mammal no diuresis occurs, whereas an immediate diuresis would follow an injection of water or KCl (Smith, 1937, p. 155).

In all fresh-water animals losses of salts are inevitable; the losses are small from *Sialis* larvae because of the relative impermeability of the cuticle. In the absence of an external ion-absorbing mechanism, these losses must be made good by absorption of salts from the food and, in these larvae, this can be associated with the well-developed mechanism for the uptake of NaCl found in the gut.

Finally, the fact that *Sialis* larvae are unable to live in saline solutions more concentrated than the blood can be explained from a knowledge of their powers of ion and water regulation. The necessary physiological mechanisms are present but are not well enough developed as, for example, they must be in *Aedes detritus* (Beadle, 1939; Ramsay, 1950). Thus drinking is necessary to make good water losses if the blood is hypotonic to the external medium; drinking does occur but only up to concentrations of the outside medium 50% greater than the blood. This mechanism resembles the drinking occurring in marine Teleosts (Smith, 1930). Since uptake of salts through the gut and diffusion through the cuticle occurs in saline solutions a hypertonic excretory fluid would have to be produced. Although the larvae are able to concentrate salts in the rectal fluid they are not able to produce a hypertonic fluid.

SUMMARY

1. The electrolyte composition of the blood, tissues and excretory fluid of the aquatic larvae of *Sialis lutaria* has been measured, and the regulation of the concentrations of sodium, potassium and chloride in the blood studied in detail.

2. In the normal larvae these ions are not present in the excretory fluid. Potassium and, perhaps, sodium are reabsorbed in the rectum but chloride is never present in the rectum.

3. If these ions are present in the outside medium they are taken into the larvae through the gut. The blood concentration is regulated by the excretion of these ions via the rectal fluid. Potassium is rapidly excreted but chloride tends to be retained in the blood. Sodium is removed more rapidly than chloride.

4. Water enters the larvae by osmosis through the cuticle, but can also be absorbed through the gut by osmosis or together with sodium ions. The water intake is balanced by excretion of rectal fluid. The factors affecting the rate of water excretion have been studied.

5. The larvae are unable to survive in hypertonic saline solutions. This is due to their inability to make good osmotic water loss or to produce a hypertonic excretory fluid.

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IONIC REGULATION IN THE MUSCLE FIBRES OF *CARCINUS MAENAS*

I. THE ELECTROLYTE COMPOSITION OF SINGLE FIBRES

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INTRODUCTION

The electrolyte composition of animal tissues, particularly the excitable tissues like nerve and muscle, have interested biologists for many years, and many theories have been advanced to explain the fact that their composition is often considerably different from that of the surrounding blood.

For the most part attention has been devoted to vertebrate cells, but in recent years the most important advances in nerve physiology have come from a study of the axons of marine invertebrates. The giant axons of cephalopods, because of their very large size, have proved particularly favourable material (see review by Hodgkin, 1951).

For a study of muscle composition and ionic equilibrium the muscles of the Decapod Crustacea offer similar advantages since the individual fibres are much larger than those in vertebrate muscle and they can be easily separated from each other. The electrical activity of these muscles has already been investigated by Fatt & Katz (1953) who find important differences from vertebrate muscle.

For the work described in this series of papers the common shore crab, *Carcinus maenas*, has been used since, as it is a marine animal, the ionic concentrations are high and this makes for more accurate analysis and also the blood concentration and the equilibrium conditions can be altered by keeping the animals in diluted sea water. Muscle fibres derived from segments of the chela are used; these fibres vary from 100 to 500 μ in diameter and are several millimetres in length.

MATERIAL AND METHODS

The crabs were supplied by the Dove Marine Laboratory, Cullercoats, where they had been collected locally, and were transferred to an aquarium into which sea water was pumped from a closed circulation system. The temperature of the aquarium water was about 17° C. and the crabs would live in these conditions for many months if they were fed at regular intervals.

The composition of the aquarium water was slightly different from that of many natural sea waters. The concentrations of some of the main inorganic constituents are shown in Table 1, and a comparison of these figures with those for other sea waters (see, for example, Prosser, 1950, p. 78, table 9) shows the potassium and

calcium concentrations to be unusually high. As will be seen later, these differences may possibly affect the blood composition of the crabs.

Blood was removed from the crabs in a pipette inserted through the arthrodial membrane at the base of one of the walking legs; it was allowed to coagulate and 0·2 ml. of the plasma was diluted to 25 ml. and this solution was used for the measurement of blood conductivity, sodium, potassium and chloride concentrations. A second 0·2 ml. blood sample was taken for the estimation of calcium and magnesium concentrations.

Table 1. *Composition of the aquarium sea water*

(Concentrations expressed as m.equiv./l.)

Conductivity (=NaCl soln.)	Na	K	Ca	Mg	Cl
610	455	12·8	28·4	112	533

Sodium and potassium were measured in solutions diluted to contain not more than 4 and 8 p.p.m. respectively, by means of an 'Eel' Flame Photometer. Chloride concentrations were measured by the Volhard back-titration method using N/100 sodium thiocyanate, and this titration method was also used for the estimation of calcium and magnesium after they had been converted quantitatively to chlorides by means of the method described by Shaw (1955). If sufficient calcium was available, this ion was also measured by the Flame Photometer in a solution containing up to 40 p.p.m. of the ion.

For this investigation the two muscles in the meropodite segment of the chela were used. These are the carpopodite extensor and flexor, and they lie more or less parallel to each other along the length of the segment. The fibres of these muscles are inserted on the inside of the shell of the meropodite and have their origin on the chitinous tendon which is attached to the junction of arthrodial membrane and the carpopodite. The extensor was used most often as the fibres are larger and longer than those of the flexor. The muscles were prepared by bisecting the meropodite longitudinally so that each part contained one of the muscles; the muscle was then removed by cutting the insertions of the fibres on the shell with a fine scalpel and finally severing the top of the tendon so that the entire muscle, together with the tendon, could be removed. The excised muscle was blotted on filter-paper to remove as much as possible of the adhering blood and then washed quickly in a solution of dextrose isotonic with the blood to remove any remaining blood. For this operation the tendon was held in forceps and the fibres agitated in the solution with a blunt needle rapidly so that the total washing time did not exceed about 30 sec. After this washing the muscle was again blotted on filter-paper to remove the dextrose solution. Now the tendon was removed by severing, with a sharp scalpel, the attachment of the muscle fibres. The muscle prepared in this way was used for the estimation of ion concentrations in the whole muscle.

The individual fibres of the muscle are large and well separated from each other so that single fibres can be easily dissected. Muscles dissected from the meropodite

as described above and washed in isotonic dextrose were transferred to liquid paraffin. The cut end of a single fibre was held in forceps, the fibre separated from the rest of the muscle and its attachment to tendon cut with fine scissors. The fibre was lifted out and blotted gently to remove liquid paraffin.

For the measurement of the electrolyte composition of the whole muscle, the prepared muscle was first weighed and then dried to constant weight to determine the water content. For cation estimations the dried muscle was incinerated in a muffle furnace at 450–500° C. until all of the organic matter had been removed. The ash was dissolved in N/10-HCl and sodium, potassium, calcium and magnesium concentrations measured in the same way as described for the blood.

Muscle chloride was measured by the Volhard method. The dried muscle was placed in a crucible, together with a known quantity of silver nitrate and about the same volume of concentrated nitric acid, and this mixture heated in a 100° C. oven until the muscle had dissolved. The presence of the silver nitrate prevented the loss of chloride which would have otherwise occurred.

Single muscle fibres, isolated for analysis as described above, were each weighed on a 5 mg. torsion balance, the average weight being about 0·5 mg. For chloride, calcium and magnesium measurements the weighed fibres were transferred to small 'Hysil' tubes, 2 cm. long and 2 mm. internal diameter. Concentrations were estimated using the ultra-micro methods described by Shaw (1955) which involves the quantitative conversion to chlorides and the estimation of the chloride by the Volhard method. Potassium estimations were made in the same way except that the incineration was carried out in very small platinum dishes and the ash subsequently washed into the 'Hysil' tubes with dilute HCl. The analyses were arranged so that the final titration measured between 0·5 and 1 μg. of chloride and the accuracy of the methods about $\pm 1\%$. Fibre chloride was titrated in the same way after digestion of the fibre in nitric acid in the presence of excess silver nitrate.

Conductivity of the blood was measured in a conventional conductivity cell which contained 5 ml. of fluid. The cell formed one arm of an impedance bridge supplied with an alternating voltage of 1500 cyc./sec. The resistance of the cell was measured when filled with a blood sample diluted to contain about 20 p.p.m. of salt.

An estimate of the conductivity of single muscle fibres was made by allowing the fibre to act as the conductivity cell itself. The electrodes were formed by small strips of platinum foil fixed on to a block of wax as shown in Fig. 1. Wires attached to the base of these strips were connected to the bridge arm. A single fibre was laid across the two electrodes and a large drop of crab Ringer solution placed on the strips at each end of the fibres to make a low resistance contact with the electrodes. The specific resistance of the fibre was calculated from measurements of the resistance of the preparation and the dimensions of the fibre (length and diameter) made to the nearest $\frac{1}{100}$ mm. by means of a travelling microscope. The method was checked by using, in the place of the fibres, pieces of glass capillary of roughly the same dimensions and containing saline solutions of known conductivity. As for the fibres, the resistance of the fluid and the length and diameter of the capillary were measured. The calculated specific resistances for two solutions are shown in Table 2.

The mean values for the two solutions of 20·6 and 40·9 agree quite well with the calculated values of 20·3 and 39·0 respectively, but the accuracy is not of a very high order. Standard deviations of ±3·3 for the Ringer solution and ±4·9 for the 50% Ringer solution are found and thus no great reliance can be placed on a single determination made by this method, but six or more measurements will reduce the error to acceptable proportions.

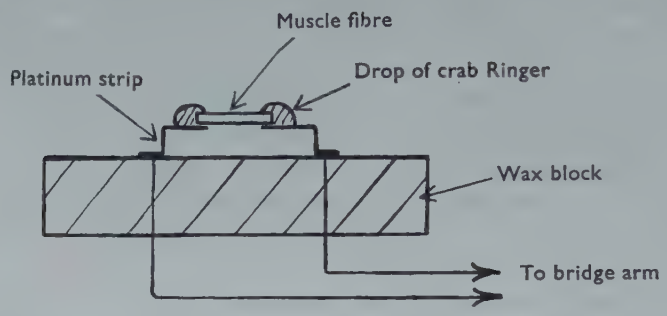

Fig. 1. The apparatus for the measurement of conductivity of single isolated muscle fibres.

Table 2. *Standardization of the fibre conductivity method: glass capillaries containing saline solutions*

Resistance (kΩ)	Capillary diam. (cm.)	Length (cm.)	Specific resistance (Ω-cm.)
	A. Crab Ringer solution		
13·8	0·028	0·350	24·3
21·0	0·022	0·468	17·1
12·0	0·022	0·254	18·0
11·8	0·026	0·324	19·3
22·2	0·018	0·298	19·0
12·7	0·032	0·412	24·8
21·6	0·028	0·568	23·4
10·7	0·024	0·292	16·6
			Mean 20·3
	B. 50% Crab Ringer solution		
51	0·022	0·448	43·2
30	0·022	0·352	32·4
30·6	0·026	0·362	44·9
21·9	0·030	0·344	44·9
43·8	0·022	0·420	37·8
33·3	0·024	0·356	42·3
			Mean 40·9

Measurements of the muscle fibre membrane potentials were made by means of intracellular electrodes of the type first used by Ling & Gerard (1949). These were constructed from 2 mm. 'Pyrex' tubing, one end of which was drawn out to a fine capillary by hand and a very fine tip being drawn on the end of this by the use of a De Fonburne Microforge. The diameter of the tip was 2 μ or less. The electrode was filled with saturated KCl solution and a silver wire coated with silver chloride inserted through the other end of the electrode, the completed electrodes having

a resistance between 2 and 10 MΩ. Then the electrode was held in a micromanipulator of the Chambers type and was used to pierce the fibre membrane; the other electrode which remained on the outside of the fibre was constructed in the same way except that the capillary at the tip was of much greater diameter and hence the resistance of the whole electrode much lower.

The fibre potentials were measured on intact carpopodite extensor muscles. The carpopodite and the meropodite were removed from one of the chelae and the shell of the meropodite cut longitudinally so that the flexor was removed leaving the extensor intact (Fatt & Katz, 1953). The preparation was fixed into a 'Perspex' box, just large enough to accommodate it, by means of small pieces of plasticine. The external electrode was held by one of these pieces so that its tip was near the muscle. The preparations was covered with blood which had been extracted from the animal previously, allowed to coagulate and the precipitate removed, and finally a thin layer of liquid paraffin was spread over the blood. The box was placed on the stage of a Greenough binocular microscope and illuminated from below. The intracellular electrode was lowered beneath the paraffin layer and watched while it was inserted transversely through the fibre membranes.

The potential difference between the two electrodes was measured by a valve millivoltmeter consisting of a pair of RCA 954 valves arranged as electrometers in a bridge circuit and the readings were made on a C.I.C. spot galvanometer connected across the two anodes. The input impedance of the apparatus was 1000 MΩ. The calibration of the apparatus and the electrodes was checked before and after each set of readings by placing the two electrodes in separate vessels each containing crab Ringer solution and then applying a variable known potential between the two vessels.

BLOOD COMPOSITION

The ionic composition of the blood of *Carcinus maenas* has been thoroughly investigated by Webb (1940). However, in view of the importance of an exact knowledge of the blood composition in relating it to the concentrations of ions found in the muscles, measurements have also been made on the blood of the crabs used in this work. Table 3 gives the summarized results of these analyses and indicates certain differences from the results obtained by Webb. The chloride concentration is

Table 3. *Blood composition*

	No. of measurements	Concentration (m.equiv./l.)	Standard deviation ±m.equiv./l.	Webb (1940) (m.equiv./kg. H_2O)
Conductivity (≡NaCl soln.)	10	552	20	—
Sodium	14	468	18	515
Potassium	23	12·1	0·9	11·9
Calcium	6	35	2·6	25·8
Magnesium	6	47·2	6·1	38·4
Sum of cations		562·3		
Chloride	24	524	18	540

slightly lower and the sodium concentration distinctly less, but on the other hand magnesium is higher and calcium very much higher (40%). Some of these differences may be due to the unusual composition of the aquarium water—for example, the high blood calcium might be related to the high calcium concentration of the latter—but it seems unlikely that the aquarium water composition would affect the concentration of sodium and chloride in the blood. The total cation concentration agrees well with the conductivity measurements which are considerably lower than those for the aquarium water (552 compared with 610). These differences are probably due to other factors such as the different localities from which the crabs were collected, their size and age, etc.

COMPOSITION OF SINGLE MUSCLE FIBRES

Measurements have been made of the potassium, chloride, calcium and magnesium concentrations in single fibres. The fibres were isolated from the muscle as described above, and then the remainder of the muscle was dried and analysed itself, so that the concentration of ions measured in the single fibres and in the prepared whole muscle could be compared. The water content of each fibre was not measured but was calculated from the wet weight of the fibre and from the water content of the whole muscle as determined from the wet and dry weights of the remainder of the muscle. This averaged 73·5% of the wet weight.

The analyses of the chloride content of thirty muscle fibres derived from the muscle from five crabs are shown in Table 4. The mean value is 53 mM./kg. of fibre water; there is little variation in the mean values for the individual crabs and the differences are not statistically significant. The variation in the chloride concentration in the individual fibres of a single crab is more marked, and since an average figure was used for the water content this may indicate either that the fibres vary in their water content or in their chloride concentration. Probably the results reflect a variability of both factors.

Table 4 also shows that there is a close correspondence between the chloride concentration as determined in single fibres and in the whole muscle, and thus indicates that the method employed for the preparation of the muscle for analysis leaves it practically free from contamination with extracellular material such as blood.

The value for the chloride content is much higher than is found in vertebrate muscle—for example, Boyle & Conway (1941) give the chloride concentration of frog's sartorius muscle as 1·2 mM./kg. H_2O—but this is to be expected in a marine organism. It may be compared with the chloride concentration found in the giant axons of cephalopods, e.g. *Loligo* axon 40 mM./kg. H_2O (see Steinbach, 1941*a*; Hodgkin, 1951, table 2).

The concentrations of potassium in the fibres, shown in Table 5, were measured on twenty-nine isolated fibres from five crabs. Again the mean concentration for individual crabs are not significantly different but the potassium concentration of the individual fibres exhibits considerable variation. The mean value of 112 mM./kg.

fibre water again is not significantly different from the value found in the analysis of the whole muscle.

It is noteworthy that although the chloride concentration is much greater than that of vertebrate muscle, the potassium concentration is of the same order. Thus Boyle & Conway (1941) give the concentrations of potassium in frog's sartorius as 125 mM./kg. H_2O, and similar figures have been found for mammalian muscles.

Table 4. *Chloride concentration of single fibres*

(mM./kg. water.)

Crab serial no.	Fibre no.	Fibre wt. (mg.)	Cl concn.	Cl concn. mean	Cl concn. whole muscle
40	1	0·90	48		
	2	0·59	54		
	3	0·34	65		
	4	0·34	59	57	50
	5	0·42	62		
	6	0·44	55		
41	1	0·65	50		
	2	1·12	72		
	3	0·69	42		
	4	0·72	55	53	60
	5	0·49	55		
	6	0·46	41		
43	1	0·70	49		
	2	0·89	46		
	3	0·91	54		
	4	0·49	47	48	47
	5	0·66	53		
	6	0·50	39		
44	1	0·85	53		
	2	0·73	58		
	3	0·45	55		
	4	0·57	60	58	50
	5	0·57	55		
	6	0·33	65		
45	1	0·69	64		
	2	1·08	48		
	3	0·46	64		
	4	1·08	36	48	—
	5	0·82	42		
	6	0·85	32		
			Mean	53	52

Concentrations of potassium in muscles of other marine animals are not well established. Steinbach (1940, 1941 *b*) gives values for Thyone muscle and Phascolosoma muscle as 169 and 106 mM./kg. tissue respectively, but these analyses include from 30 to 40% extracellular material. If corrected for this and for the water content the values expressed in mM./kg. H_2O would be considerably greater than found in *Carcinus* muscle. In the nerve fibres of several marine animals the analyses are more certain and all show very high potassiun concentrations—for example the giant axons of *Loligo* (Steinbach & Spiegelman, 1943; Keynes & Lewis, 1951 *a*), the axons of *Sepia* (Keynes & Lewis, 1951 *a*) and the nerve fibres of *Carcinus* (Keynes

& Lewis, 1951*b*) have potassium concentrations between 330 and 460 mM./kg. water. It would appear that this difference in potassium concentration between that found by Keynes & Lewis for *Carcinus* nerve, and that reported in this paper for the muscle must be associated with some functional difference in the behaviour of the ions in the two different cell types.

Table 5. *Potassium concentration of single fibres*

(mM./kg. water.)

Crab serial no.	Fibre no.	Fibre wt. (mg.)	K concn.	K concn. mean	K concn. whole muscle
43	1	0·69	112		
	2	0·65	63		
	3	0·39	147	117	104
	4	1·12	109		
	5	0·72	155		
44	1	1·90	99		
	2	1·46	106		
	3	2·08	112		
	4	1·18	122	111	—
	5	0·75	113		
	6	2·00	113		
45	1	1·34	111		
	2	1·83	111		
	3	0·90	139		
	4	1·38	122	114	118
	5	1·72	74		
	6	1·15	125		
46	1	1·88	108		
	2	0·49	115		
	3	0·92	126		
	4	1·21	109	108	124
	5	2·10	107		
	6	2·04	85		
47	1	0·92	89		
	2	0·69	120		
	3	0·77	129		
	4	0·94	105	112	124
	5	0·72	114		
	6	0·67	115		
			Mean	112	117

The concentrations of calcium and magnesium were determined together on the isolated fibres. Owing to the low concentration of calcium present, in most cases several fibres (generally about six) were isolated, and used together for each determination. This can be seen in the weight records which are in general much greater than for single fibres. Table 6 shows the results for twenty-five groups of fibres derived from five crabs. The mean value for calcium is 5·2 mM./kg. water and for magnesium 16·9. These values do not differ much from the concentrations found by Boyle & Conway for the frog's sartorius muscle 3·3 and 16·7, although in *Carcinus* these concentrations are lower than the respective concentrations in the blood, while the opposite is the case in the frog.

Table 6. *Calcium and magnesium concentrations in single fibres*

(mM./kg. water.)

Crab serial no.	Fibre no.	Fibre wt. (mg.)	Ca concn.	Whole muscle Ca concn.	Mg concn.	Whole muscle Mg concn.
46	1	1·52	3·0	—	—	—
	2	0·76	4·7	—	—	—
	3	1·56	1·9	—	—	—
	4	1·12	3·5	—	—	—
	5	0·64	6·5	—	—	—
	6	1·69	2·7	—	—	—
		Mean	3·7	6·1	—	—
47	1	8·03	3·6	—	16	—
	2	7·69	6·6	—	17	—
	3	7·50	6·0	—	13	—
	4	5·85	—	—	19	—
	5	8·36	5·4	—	14	—
		Mean	5·4	5·9	16	17·3
48	1	5·47	4·7	—	18·6	—
	2	4·99	5·1	—	14·7	—
	3	4·38	4·7	—	17·6	—
	4	4·87	5·8	—	15·0	—
		Mean	5·1	7·1	16·5	18·7
49	1	4·15	5·6	—	21·0	—
	2	4·44	5·8	—	17·4	—
	3	4·69	5·0	—	17·5	—
	4	3·51	4·6	—	16·2	—
		Mean	5·2	7·7	17·8	17·5
50	1	3·46	6·6	—	21·0	—
	2	3·43	11·9	—	17·3	—
	3	4·12	5·6	—	12·2	—
	4	2·44	4·6	—	17·3	—
	5	2·91	5·3	—	19·6	—
	6	3·84	6·0	—	16·6	—
		Mean	6·7	7·1	17·3	23·1

COMPOSITION OF THE WHOLE MUSCLE

It has been seen in the previous paragraph that the results of the single fibre analysis are not significantly different from those found for the whole muscle which had been prepared by careful washing. Although losses of ions from the muscle would almost certainly occur in isotonic sugar solutions (e.g. Steinbach, 1941*a*) the washing was always rapid (less that 30 sec.), and it is not likely that much change would have occurred during this period. The summarized results for all the measurements made on the whole muscles are shown in Table 7 which includes estimations of sodium concentration, which were not made on the single fibres. However, in the latter case, in view of the close correspondence between the analysis for single fibres and the whole muscle for the other ions, there is little doubt that these analyses do represent more or less truly the concentration of sodium found in the muscle fibres. Table 7 shows the total cation concentration to be 224 m.equiv./kg. H_2O, and conductivity measurements (see below) indicate that there is no reason to suppose that any other cations exist in the fibre water. This figure is much less than half the

corresponding figure for the blood, and if it can be assumed that the muscle fibres are in osmotic equilibrium with the blood then a large number of unionized molecules must also be present. This is not unusual among marine animals—a fact that was emphasized 50 years ago by Fredericq (1904). It is interesting in this connexion that recently Camien, Sarlet, Duchateau & Florkin (1951) found in the muscles of lobsters considerable quantities of free amino-acids of which proline, glycine and arginine were present in concentrations greater than 1%.

Table 7. *The electrolyte composition of whole muscles*

	No. of measurements	Concentration (m.equiv./kg. H_2O)	Standard deviation ± m.equiv./kg.	Mean concentration for single fibres (m.equiv./kg.)
Sodium	12	54	7	—
Potassium	13	120	14	112
Calcium	6	13·8	1·6	10·4
Magnesium	9	35·8	5·6	33·8
Sum of cations	—	223·6	—	—
Chloride	12	54	8	53

The difference between the total cation concentration and the chloride concentration (which was the only anion measured) is very marked, the deficit in the anion fraction being 170 m.equiv./kg. Some of this will be accounted for by other inorganic anions such as bicarbonate, sulphate and phosphate, but the majority must be as organic anions. Whether this fraction consists of protein and organic phosphate compounds alone or whether other organic acids such as the acidic amino-acids are present remains to be discovered.

CONDUCTIVITY OF SINGLE FIBRES

Conductivity of thirty isolated fibres from four different crabs was measured and the results are shown in Table 8. As pointed out earlier, no significance could be attached to individual results and these are therefore not given. The agreement in the results for the four crabs is good, but the variability in the apparent conductivity of the fibres of one crab is greater than found in the test experiments with glass capillaries. This may, to some extent, express true differences in the individual fibres, but may also be due to the difficulty of assessing accurately the true dimensions of the fibres due to changes in diameter along the length, which were often found.

Table 8. *Conductivity of single fibres*

Crab serial no.	No. of fibres measured	Specific resistance (Ω-cm.)	S.D. ± Ω-cm.
36	10	53	12
37	7	60	18
38	8	55	11
39	5	57	10
		Mean 56	

To interpret the mean value for the fibre specific resistance exactly in terms of ionic concentrations is not possible until the nature of all the muscle ions and their ionic mobilities are known, but an approximation can be made.

The specific resistance of a simple strong electrolyte

$$= \frac{1000}{\Lambda C},$$

where Λ is the equivalent conductance of the solution and C its concentration in g.equiv./l. Now the equivalent conductance of a solution of KCl of approximately the same concentration as the muscle ions is 110·5 (0·2 g.equiv./l. at 18° C.), whereas that of a NaCl solution of the same strength would be 87·5. Thus for a specific resistance of 56 Ω-cm., $c = 1000/110{\cdot}5 \times 56 = 161$ m.equiv./l. for KCl and $c = 1000/87{\cdot}5 \times 56 = 204$ m.equiv./l. for NaCl.

Now the concentration will not be as low as 161 m.equiv./l. since ions of lower mobility than potassium (e.g. sodium ions) are present, but on the other hand is not likely to be greater than the concentration of the NaCl solution. Now the analyses given in the previous section showed that the total concentration of cations was 224 m.equiv./l. and thus the conductivity measurements indicate that more than 72% of these cations are present in an ionized form and possibly as much as 91%. The divalent ions, calcium and magnesium, account for about 22% of the cations, and in view of known facts that these ions tend to form undissociated compounds with proteins and other large molecules it is possible that 50% or more of these molecules may not contribute to the conductance of the fibres.

MEMBRANE POTENTIALS OF SINGLE FIBRES

In order to be able to interpret the differences in concentration of the ions in the muscle fibres and in the blood measurements have been made of the potential difference across the fibre membrane which separates the two. A comparison is then possible between this potential and the calculated equilibrium potentials. The membrane potentials of a very large number of fibres were measured on healthy and active crabs, and the results are summarized in Table 9. The consistency of the results is good, although lower potentials have been recorded in less active crabs.

Table 9. *Muscle fibre potentials*

(Inside of the fibre negative; temp. = 17° C.)

Crab serial no.	No. of fibres measured	Potential (mV.)	S.D. ±mV.
27	33	60	5
28	14	61	3
29	12	59	3
30	16	53	7
32	22	60	5
33	14	59	7
34	23	57	5
35	15	55	6
		Mean 58	

The mean value of 58 mV. is somewhat lower than recorded by Fatt & Katz (1953) for the fibres of *Portunus depurator*, and occasionally *Carcinus maenas*, for which they found a mean value of 70 mV. This difference may be due to several factors, such as the slightly lower temperature, the different species used for most of the experiments and the different physiological conditions (Fatt & Katz used a crab Ringer solution rather than the animals' own blood).

The potentials recorded in this paper for *Carcinus* muscle are much lower than those found in vertebrate muscle using the same micro-electrode technique (see Hodgkin, 1951, table 1), where potentials of around 80 or 90 mV. have been recorded but resembles more the potentials found in the cephalopod axons.

Now if the membrane is freely permeable to a particular ion and the fibre is in equilibrium with the blood then the concentration of this ion (more properly, its activity) in the blood is related to that in the fibre in the following way:

$$C_i = C_o \exp \frac{nF}{RT}(E_o - E_i) \text{ for a cation,}$$

and

$$A_i = A_o \exp \frac{nF}{RT}(E_i - E_o) \text{ for an anion,}$$

where C_i and A_i are the concentrations in the fibre and C_o and C_i the blood concentrations; n is the valency; $E_o - E_i$ is the equilibrium potential difference across the membrane in volts; F is the Faraday; R is the gas constant and T the absolute temperature.

Table 10 shows the values of the equilibrium potentials calculated from the ratios of the ions found in blood and muscle and also the observed membrane potentials. The equilibrium potentials have been calculated for all the ions using the mean values from Tables 3 and 7. In addition, in some experiments membrane potentials and potassium and chloride concentrations were measured in the same crab, and these are also given in Table 10, together with the calculated equilibrium potentials.

Table 10. *The ratios of ion concentrations in the blood and fibres and the ion equilibrium potentials*

	$\frac{Cl_o}{Cl_i}$	$\frac{K_i}{K_o}$	$\frac{Na_i}{Na_o}$	$\frac{Ca_i}{Ca_o}$	$\frac{Mg_i}{Mg_o}$	Membrane potential	Equilibrium potentials				
							Cl	K	Na	Ca	Mg
Mean values	9·70	9·92	0·11	0·39	0·76	58	57·2	57·8	−54·3	−11·7	−3·5
Crab no. 30	8·69	7·81	—	—	—	53	54·5	51·7	—	—	—
31	6·53	6·26	—	—	—	46	44·3	46·2	—	—	—
32	9·68	8·89	—	—	—	60	58·9	57·4	—	—	—
33	10·26	12·09	—	—	—	59	58·8	62·7	—	—	—
34	7·72	10·91	—	—	—	57	51·5	60·1	—	—	—

In the case of potassium and chloride the correspondence between the equilibrium potentials and the measured membrane potential is very close both for the mean values and for the individual cases. Crab 31 should be noted since in this case the crab was not very active and the membrane potential was low, and it is seen that the

equilibrium potentials are also correspondingly low. There is thus no necessity to postulate any activity on the part of the fibre or its membrane in maintaining the concentrations of these ions which are found in the fibre.

With the other cations the situation is different, for sodium, calcium and magnesium all require equilibrium potentials of the opposite direction from the observed membrane potentials. In the case of the divalent ions, since there is doubt if they are present in a completely ionized form and since nothing is known of the permeability of the membrane to these molecules, no deductions can be drawn other than that if any equilibrium does exist between those in the fibre and the blood it is certainly not a simple one.

With sodium the position is somewhat clearer since it has been invariably found that sodium ions are present in low concentrations in muscles and nerves of both vertebrate and invertebrate animals. In those cases where the penetration of the muscle by sodium has been measured using radio-active sodium as a tracer the fibre membrane has always been found to be permeable (e.g. frog's sartorius; Levi & Ussing, 1948; Harris & Burn, 1949; rat gastrocnemius muscle, Heppel, 1939; *Sepia* axons, Keynes, 1951), and although measurements have not yet been made on *Carcinus* muscle it seems likely that the same will be found here. This means that the low concentration sodium ion in the muscle fibres must be maintained by some active process such as the sodium extrusion mechanism first suggested for vertebrate muscle by Dean (1941).

DISCUSSION

Boyle & Conway (1941) proposed, as a result of their experiments on the frog's sartorius muscle, that the muscle fibre membrane was permeable to the smaller ions such as potassium and chloride but impermeable to larger ones like sodium and organic anions. They further suggested that the distribution of the potassium and chloride ions was the result of a Donnan equilibrium which existed across the fibre membrane as a result of the high concentration of sodium ions on the blood side of the membrane and the large number of organic anions in the fibre. The use of radioactive sodium has shown that the fibre membrane is in fact permeable to sodium ions, and in consequence this theory has had to be modified to take into account this fact. Dean (1941) proposed that sodium ions were actively extruded from the fibre and that this 'sodium pump' balanced the intake of sodium ions by diffusion.

The results obtained for the muscle fibres of *Carcinus*, as far as they go, are in complete agreement with the predictions of Conway's theory, despite the fact that the ionic concentrations both in the blood and in the fibres are, in most cases, totally different from those found in vertebrate muscle. Whether or not this agreement is a real one and not merely coincidental will be shown by the composition of the muscles under conditions of varying blood concentration. It is under these conditions that it can be seen to what extent the fibre behaves in a passive manner or actively regulates the many variable factors, such as ion concentrations, water content and membrane potential.

SUMMARY

1. Measurements have been made of the concentrations of potassium, chloride, calcium and magnesium, the conductivity and the membrane potential of single isolated fibres of the carpopodite extensor and flexor muscles of *Carcinus maenas*.

2. Analyses of whole muscles gave the total concentration of the cations as 224 mM./kg. H_2O, of which potassium accounted for 120 mM./kg. and sodium 54 mM./kg. Of the anion fraction chloride only accounted for 54 mM./kg. H_2O. The analyses of the separated fibres were the same as for the whole muscle.

3. The average specific resistance of the fibres is 56 Ω-cm. This represents a concentration of muscle ions of about 200 m.equiv./kg. and the electrolyte content of the muscle is not much more than a third of that of the blood. Between 72 and 91% of the total muscle fibre cations are present in an ionized form.

4. The average membrane potential is 58 mV. The ratios of the concentrations of potassium ions and chloride ions in the blood and muscle fibres suggest that these ions may be passively distributed across the membrane. The low concentration of sodium ions in the fibre probably indicates the operation of a 'sodium pump' as has been proposed for vertebrate muscles. The distribution of calcium and magnesium cannot be explained in simple terms.

5. The correspondence between the equilibrium potentials for potassium and chloride ions and the membrane potential suggests that theory of ion distribution put forward by Boyle & Conway for frog's sartorius muscle may also be applicable to *Carcinus* muscles.

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FUNCTIONING OF THE INSECT OCELLAR NERVE

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INTRODUCTION

In a paper on the function of the ocelli of the African migratory locust, *Locusta migratoria migratorioides* R. & F., Parry (1947) stated that 'when the ocellus is darkened, the electrical response in the nerve consists of a decrease in potential near the ocellus, relative to the cut end. No impulses were detected in the ocellar nerve'. Instead of nerve impulses he suggested that 'darkening the ocellus causes a depolarization which spreads down the ocellar nerve and depolarizes a ganglion in the brain, thereby inducing the discharge of impulses down the commissures'. This type of phenomenon has not been observed elsewhere but would, if substantiated, be of major importance for any general theory of nervous function. Parry's exposition has already received acceptance in the major text-books of insect physiology (Wigglesworth, 1953; Roeder, 1953). The decrease in potential was observed to be initiated not by a stimulus (light) but by the cessation of a stimulus (darkening). This also constitutes a unique situation, since all other light-sensitive organs show a depolarization on illumination (Granit, 1950).

Following Adrian's demonstration of a slow potential change preceding bursts of nerve impulses in the isolated nerve cord of *Dytiscus* (Adrian, 1931) there has been a general belief among comparative physiologists that small internal gradients of potential difference are important in the functioning of insect central nervous systems (e.g. Hughes, 1952). The locust ocellar nerve, if it functions in the way Parry suggests, would provide an excellent preparation for the study of such potential gradients and their effects in insect C.N.S. In view of the serious divergencies between the potentially important scheme proposed by Parry for the ocellar nerve, and current concepts of nervous function, it was thought desirable to re-investigate the ocellar nerve preparation.

With the introduction of intracellular microelectrodes the general physiologist has been provided with an excellent tool which should be capable of resolving this kind of problem, by providing unambiguous information about the functioning of individual fibres. According to Parry the median ocellar nerve contains four very large nerve fibres of about 25 μ diameter. The outside diameter of the whole nerve (author's measurements) is about 65 μ, so following removal of part of the nerve sheath, penetration of these axons with an intracellular electrode should not be difficult. However, in pursuing the problem with this method in preliminary studies, using the median ocellar nerve of *Locusta*, I was not able to obtain penetrations consistent with this picture. The difficulty was sufficient to warrant a separate examination of the histology of the median ocellar nerve.

HISTOLOGY OF THE MEDIAN OCELLAR NERVE

Ocellar nerves were dissected out from two animals, one fixed in Duboscq's fixative and the other in Bouin. They were embedded in paraffin wax, sectioned at 5 μ and stained with Van Gieson (this work was done by Mr D. W. Wood). The whole nerve trunk is surrounded by the usual thick neural lamella, but no 25 μ axons were observable inside it. There are two axons of 8 μ which are nevertheless 'giants' by locust standards; the smaller 'giant' nerve fibres of the nerve cord are also of this order of magnitude (Cook, 1951). There are four other axons, each 4 μ in diameter. The combined axons occupy only a small part of the total volume of the nerve cylinder. The rest of the space is occupied by cellular material similar to the sheath cells, together with some fluid.

CONSIDERATIONS FOR ELECTRICAL RECORDING

The small size of the constituent nerve fibres would make it extremely difficult to utilize intracellular recording electrodes in studying their function and with six nerve fibres present it would, in any case, be desirable to record their activity together. This necessitates the use of external electrodes, but the large amount of fluid-filled space in the trunk must be expected to provide a considerable short-circuit for any electrical activity which may be present in the nerve fibres. However, since the nerve is at least 1 mm. long in an adult locust, external recording, even of action potentials should certainly be possible. Some degree of drying out of the nerve should greatly facilitate recording, by increasing the resistance of the material short-circuiting electrical activity in the nerve fibres. Parry took every precaution to *prevent* any drying out of the nerve.

METHODS

Isolated heads of *L. migratoria* were embedded in plasticine and dissected from the left side in order to expose the nerve of the median ocellus; this was completely freed from any surrounding tissue. The circumoesophageal commissure on the left side was also exposed. The antennal nerves were then cut and the lateral ocellus and compound eye on the exposed side covered with plasticine. The preparation was then bathed in the author's locust saline (Hoyle, 1953). Two hook electrodes were manipulated into position on the ocellar nerve with the aid of an assembly of Palmer blocks. The electrode nearest to the ocellus was made of silver wire tapered by anodal dissolution in dilute silver nitrate solution to a diameter of about 25 μ. It was insulated with polythene which was melted over the wire under a binocular microscope so as to leave a small piece of the hook bared. This fine wire tended to cut into the nerve and therefore to establish fairly good electrical contact with the fibres. The second electrode was also made of tapered silver wire, but was made somewhat thicker (about 40 μ) in order to support the nerve; it was placed nearer the brain. The first electrode was connected to the input grid of a direct-coupled amplifier (Pye) with a single-sided input, the second electrode being connected to

the cathode. A second pair of electrodes with much larger hooks was placed in contact with the circumoesophageal nerve and action potentials led off through a condenser-coupled amplifier with balanced input. Two methods of display were used, connected in parallel: a double-beam oscilloscope (Cossor 1049) and a pen recorder (Ediswan E.P.R.). The latter instrument offers the advantages that the nerve response can immediately be ascertained, avoiding the delay involved in developing lengths of photographic paper, and that complete records of a long experiment can be made at small cost. It suffers from the disadvantage that its maximum rate of writing is only 90–100 cyc./sec. For the present purpose this was not a serious disadvantage and most of the records displayed in this paper were obtained with this instrument. The pens were not of equal length so coincidence points have been drawn as small upwards or downwards traces on each record and labelled C.P.

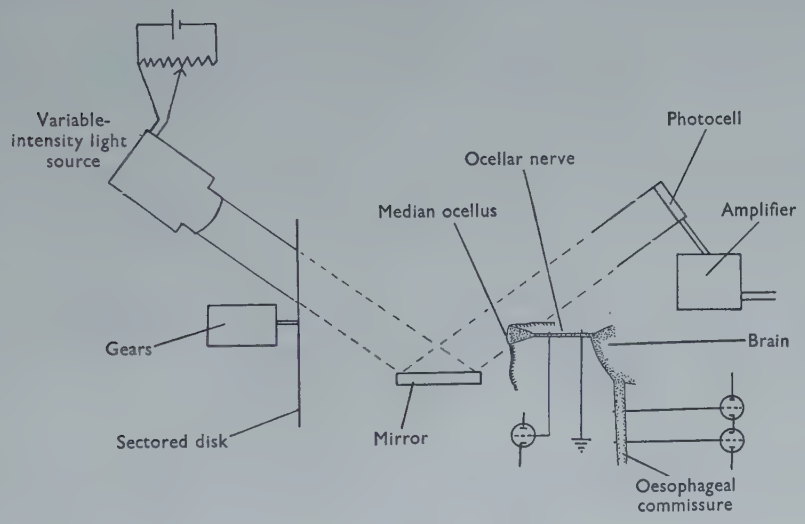

Fig. 1. Diagram of the preparation and experimental arrangement. The head was dissected from the left side.

A straight external electrode was also available, as an alternative to the finer hook electrode, for recording the retinal potential. The ocellus was illuminated from a microscope lamp having a variable-intensity control. The light beam was directed upon the median ocellus by a small mirror (Fig. 1), the part of the beam not interrupted by the preparation being allowed to strike a barrier-layer photocell which monitored the light stimuli. The photocell did not give an accurate indication of the light stimulus and was only used to indicate the timing of the light changes. This arrangement of illumination from below the preparation prevented light falling on the electrodes and leading to the production of a photoelectric effect there. The light beam could be interrupted by means of a sectored disk, driven by hand through an assembly of gears.

RESULTS

The retinal potentials (E.R.G.)

In order to study the retinal potentials the straight electrode was placed with its tip at the back of the retina and the larger hook electrode of the ocellar nerve pair somewhere in the saline pool. On illumination of the ocellus the retinal lead shows a large, simple, monophasic change of potential which is negative with respect to the electrode in the saline (Fig. 2). On cessation of illumination a potential wave of similar shape but reverse sign and smaller magnitude is recorded. These potentials constitute a simple form of electroretinogram. Similar, though smaller potential changes can be recorded from a number of different situations in the preparation when the median ocellus is illuminated or darkened. These are due to the general flow of current in the conducting parts of the medium—a 'volume conductor'—from the dipole sources in the retina, and in most positions are opposite in sign to

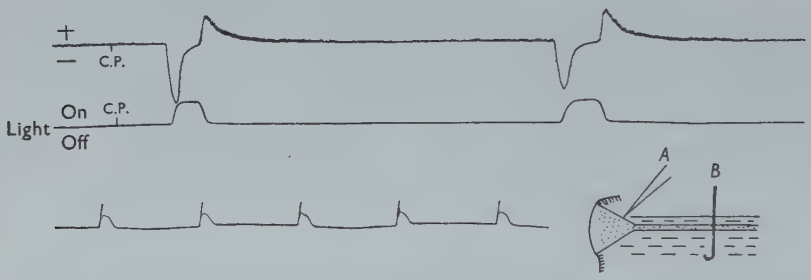

Fig. 2. The retinal potentials recorded from the back of the ocellus. The electrode arrangement is indicated in the inset diagram. Upward deflexion indicates *A* going positive with respect to *B*. Upper trace, retinal potential; middle trace, photocell response, upward deflexion indicates light on; lower trace, time in sec. Coincidence points indicated C.P.

those recorded at the back of the retina. A similar phenomenon is observed in regard to insect muscle action potentials. These are commonly recorded as positive in sign with respect to an 'indifferent' electrode when using external electrodes (Roeder & Weiant, 1950; Hagiwara, 1953). If, however, the electrode is sufficiently small and well-insulated and placed close to a muscle-fibre surface, the sign of the recorded action potential is most frequently negative (Hoyle, 1955). The positive potentials are due to the nature of the current flow in the 'volume conductor' and are much too complex to permit of a simple analysis. There are similar implications for the ocellar nerve preparation. Only an electrode of suitable type, closely applied to the retinal surface, records the negative potential; grosser and more distantly placed electrodes record the same potentials as positive in sign.

In the present experiments the retinal potentials could be picked up by the fine hook electrodes when they were in position on the ocellar nerve (Fig. 3), especially when this was bathed in the saline, and they were opposite in sign to those recorded in Fig. 2. When the nerve is in air they are more commonly recorded with the same polarity as in Fig. 2. They are not affected by crushing the ocellar nerve and so are not due to depolarization spreading along the nerve fibres. The reversed retinal

potential changes recorded at the nerve electrodes in saline are similar in sign and shape to the potentials recorded from the ocellar nerve by Parry. In his experiments the nerve was surrounded by medicinal paraffin 'saturated with oxygenated Ringer'. An examination of Parry's results (1947, fig. 2, p. 215) shows that an electrode near to the cut end of the nerve always went negative with respect either to another electrode placed on the nerve nearer the ocellus, or to an indifferent

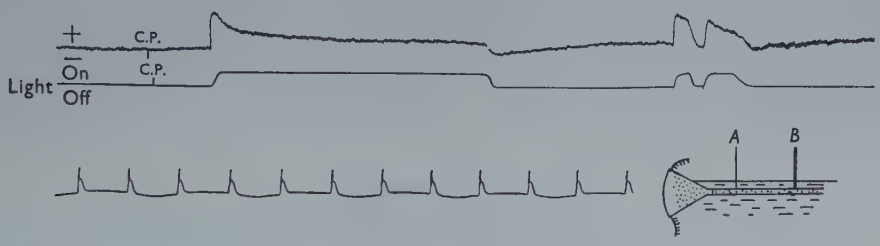

Fig. 3. The retinal potentials recorded at the ocellar nerve bathed in saline. The arrangement of the electrodes is indicated in the inset diagram. Upward deflexion indicates *A* going positive with respect to *B*. Upper trace retinal potentials; middle trace, photocell response, upward deflexion indicates light on; lower trace, time in sec. Coincidence points indicated C.P.

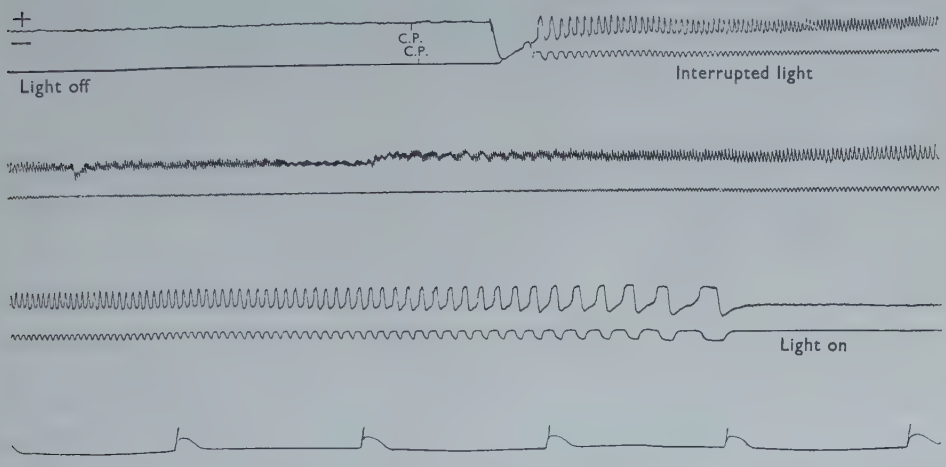

Fig. 4. Continuous record of the retinal potential changes following interruption of the light beam. Upper trace, retinal potentials recorded as in Fig. 2, positive upwards; lower trace photocell response, upward deflexion indicates light on; time in sec. Coincidence points indicated C.P.

electrode 'on any tissue in the head'. The two records are identical in shape so the two situations for the second electrode are exactly equivalent. Theoretically the potentials recorded by Parry could be obtained with either of two sources of potential difference: in the nerve between the electrodes (as he supposed), or in the ocellus. It would seem reasonable, with his recording arrangement (balanced input), to suppose that the retinal potentials, since they should constitute 'in-phase' signals would not be well recorded. On the other hand, the exact similarity between his recording with the distal lead placed first in contact with the nerve and then 'on

any tissue in the head' makes it probable that good conditions for balanced input recording had not been achieved and that the distal lead was, in effect, a grounded lead in both cases. This alters the position completely and provides a situation in which recording of the retinal potentials could easily be obtained. The proximal lead in Parry's experiments, as mentioned earlier, went negative for illumination of the ocellus, positive for darkening, a similar response to that obtained in the present work with an electrode placed at the back of the retina. All this makes it seem quite possible that Parry actually recorded the retinal potentials only.

The retinal potential changes follow changes in light intensity (interrupted light) up to a frequency of about 120 flashes per sec. (Fig. 4), although they are minute at this frequency.

Nerve action potentials

The nerve was not cut near the brain in these experiments as it was in Parry's, for in the author's experience deterioration of the nerve over such short distances as are involved in this preparation would occur almost immediately following section. Also, the depolarization of the axons, spreading from the cut ends electrotonically, would involve an appreciable part of the short length of nerve available for recording even if there were no deterioration. In order to provide optimal conditions for recording electrical activity from the nerve the surrounding saline is first sucked away using a pipette. When the nerve is in air the electrodes still pick up the retinal potentials but these are usually quite small. Now, however, conducted action potentials are evident in the nerve following changes of illumination. They cease abruptly if the nerve is crushed close to the ocellus. As the nerve dries out the pick-up of the retinal potentials is reduced and eventually disappears. At the same time the nerve action potentials become much more satisfactorily recorded. The nerve responses to changes in illumination can be grouped into three distinct categories.

(*a*) *The dark-discharge.* In darkness or very dim light there is a continuous discharge in the nerve which probably involves all the smaller nerve fibres (Fig. 5). This discharge ceases immediately the ocellus is illuminated. It persists apparently indefinitely (within the limits imposed by the life of the preparation). In light of fluctuating, fairly low intensity, the discharge shows considerable variation.

(*b*) *The 'on'-discharge.* A small, sudden increase in illumination of the ocellus, whether dark-adapted or not, leads to a large, single action potential (Figs. 5–7). The height of the smallest action potential obtained by sudden illumination is very much greater than that of the largest single action potential of the dark-discharge. This suggests that the minimum 'on'-discharge is due to a single impulse in one of the two 'giant' fibres. A larger sudden increase in illumination still produces only a single action potential, but a much larger one. This could be due to the almost simultaneous firing of the second 'giant' together with the first. There is never any repetitive discharge of these large spikes. They are evoked in interrupted light at frequencies up to 100 per sec without failures. At higher frequencies there are frequent failures and no responses at all above about 120 flashes per sec.

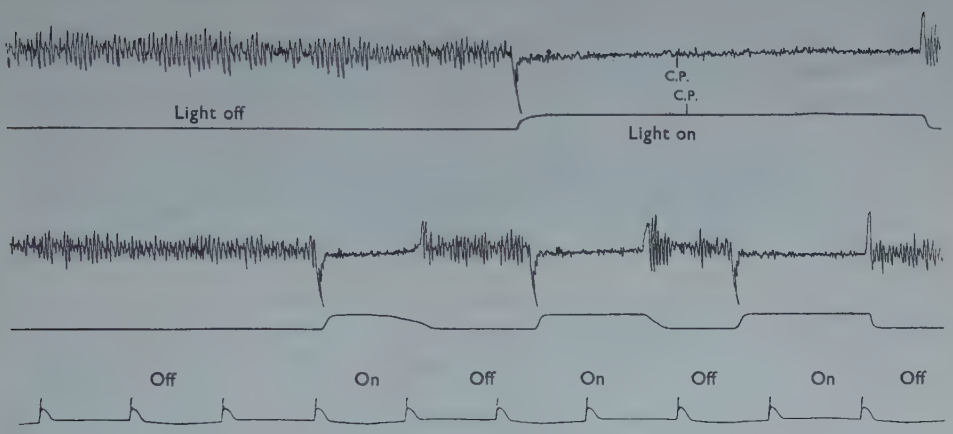

Fig. 5. Continuous record of the 'off'-discharge recorded from the ocellar nerve, and the effect of suddenly illuminating the ocellus with a light of low intensity. Note that there was an attenuated component of the retinal potentials in these records and that their sign was similar to that recorded with an electrode at the back of the ocellus. Note also the large spike which immediately follows illumination. Upward deflexion indicates electrode proximal to ocellus going positive. Upper trace, record from ocellar nerve; lower trace, photocell response, upward deflexion indicates light on; time in sec. Coincidence points indicated C.P.

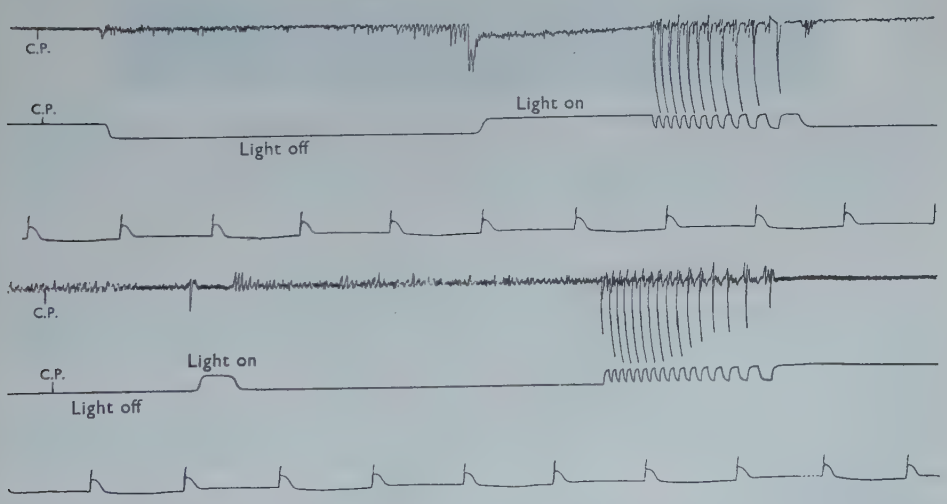

Fig. 6. Records from the ocellar nerve showing the 'dark-discharge' and the effect of interrupted light. Note the large spikes at 'light-on' and especially at the end of the records the brief burst of impulses at 'light-off'. Upper trace, record from ocellar nerve; lower trace, photocell response, upward deflexion indicates light on; time in sec. Coincidence points indicated C.P.

(*c*) *The 'off'-discharge.* A sudden decrease in illumination of the ocellus, whether light-adapted or not, leads to a very brief burst of activity in the smaller fibres (Figs. 6, 7). In fairly weak light in which the nerve is silent, a sudden decrease in illumination leads to single firing in one or two axons (Fig. 8). The 'off'-discharge apparently ceases in interrupted light at about 30 flashes per sec. (Fig. 7).

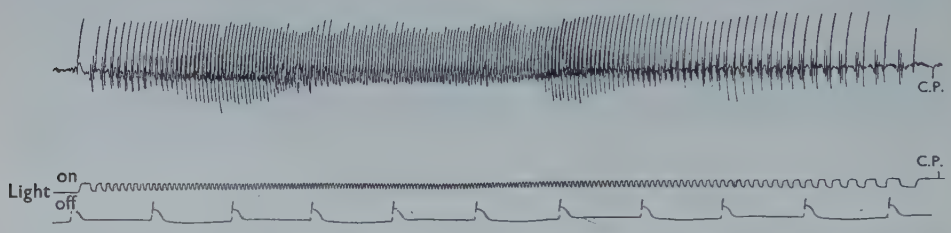

Fig. 7. Record from ocellar nerve during interruption of the light beam. Note that the spikes recorded at the 'light-off' signals have a conspicuous phase below the base-line and that this disappears at 28 flashes per sec., indicating flicker-fusion of the 'off'-discharge at this frequency. Upper trace, record from ocellar nerve; middle trace, photocell response, upward deflexion indicates light on; time in sec. Coincidence points indicated C.P.

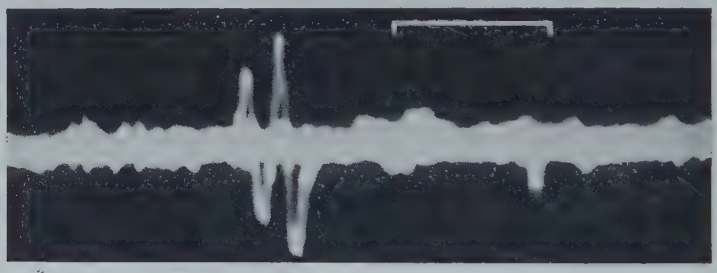

Fig. 8. Oscillograph record, using high-velocity repetitive sweeps, of a pair of propagated nerve impulses produced by a sudden darkening of the ocellus from dim lighting. Upward deflexion positive. The impulses were triphasic ones which are similar in shape to nerve action potentials recorded from other locust nerves with the present type of recording arrangement. Time trace 10 msec.

The nervous response in the commissure

Nerve action potentials are at all times easily recordable from the commissures. Three kinds of response can be observed in the commissural nerve following the discharge along the ocellar nerve.

(*a*) *The dark-discharge.* In darkness there is a continuous discharge of impulses which is greatly reduced during illumination of the ocellus (Fig. 9). The discharge is most irregular.

(*b*) *The 'on'-discharge.* A sudden increase in illumination of the ocellus leads to a brief discharge of impulses (Fig. 9).

(*c*) *The 'off'-discharge.* A sudden decrease in illumination of the ocellus leads to a brief discharge which is quite distinguishable from the general dark-discharge, but followed closely by it. In interrupted light there is an enormous increase in activity in the commissural nerve, at frequencies up to about 30 flashes per sec.

Above this frequency the discharge quietens abruptly (Fig. 10), and resembles that present in continuous light, although giant spikes of the ocellar nerve 'on'-discharge are still reaching the brain.

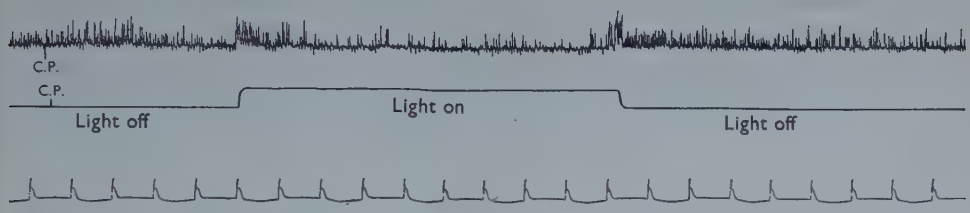

Fig. 9. Record from the left circumoesophageal commissure during darkening and illumination of the ocellus. The discharge of impulses is considerably reduced in the light. A brief burst of impulses is evident at 'light-on' and also at 'light-off'. Upper trace, record from ocellar nerve; middle trace, photocell response, upward deflexion indicates light on; time in sec. Coincidence points indicated C.P.

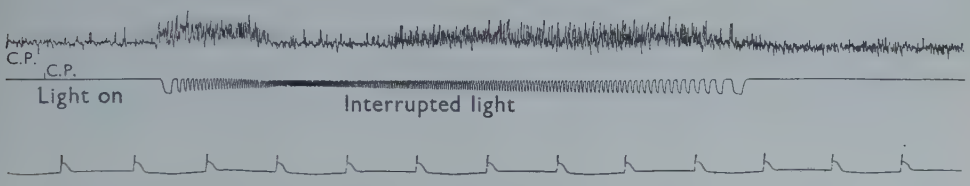

Fig. 10. Record from the left circumoesophageal commissure during interruption of the light beam. Note the considerable reduction of the discharge, to a level similar to that obtained in uninterrupted light, when the beam is interrupted at about 30 times per sec. Upper trace, record from circumoesophageal nerve; middle trace; photocell response, upward deflexion indicates light on; lower trace, time in sec. Coincidence points indicated C.P.

DISCUSSION

It is evident from the results described above that Parry's explanation of the functioning of the insect ocellar nerve must be discarded. His conclusions were based largely on three observations: the absence of nerve impulses in the ocellar nerve, the presence of a wave of depolarization in response to darkening of the ocellus and the presence of four 25 μ axons in the ocellar nerve. In the present work none of these observations has been confirmed, and it is suggested that some of Parry's observations may be attributable to the experimental procedure which he adopted, particularly the use of 'paraffin saturated with oxygenated Ringer' as a medium in which to bathe the nerve during recording. The nerve impulses present are not large, and several factors combine to make it difficult to record them: the presence of the retinal potentials, the short length of the nerve, the short-circuiting effect of inter-axon fluid and of the extensive sheath cells. A record which illustrates the difficulties is shown in Fig. 11. Propagated action potentials are, however, undoubtedly present, and entirely explain the conduction of excitation to the brain. It has been shown that the 'wave of depolarization' described by Parry could be directly due to the retinal potentials, recorded as the result of current flow in the conducting medium of which both the retina and the ocellar nerve form a part. The third point, about 25 μ axons, cannot be resolved. Six axons are present in the

sections of the median ocellus prepared during the present investigation; the two largest ones are only 8 μ in diameter.

Until very recently it was also believed that nerve impulses were not present in the ocellar nerve of *Limulus*, but they have now been conclusively demonstrated by Waterman (1953) by improved recording technique.

From the results of the present work little further information can be added to the interpretation of the function of the ocellus given by Parry. It seems evident that it is concerned principally with registering sudden changes of light intensity, especially shadows. It could also act as an indicator of light intensity in dim light and could be used as an instrument of orientation in a light beam.

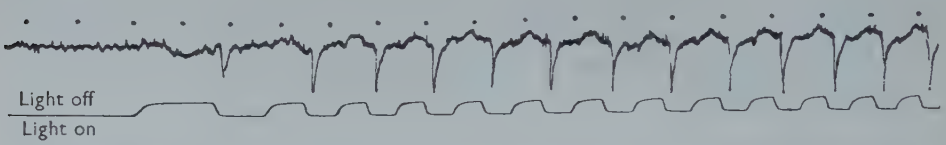

Fig. 11. Oscillograph record from ocellar nerve at the start of a test using high amplification (down to 'noise-level'). Note the conspicuous component of the retinal potentials and the lack of clear recording of any nerve responses other than the giant spikes following 'light-on'. The latency of the response is clearly visible, being 10 msec. Upper trace, record from ocellar nerve; lower trace, photocell response; time trace, 100 msec. Retouched.

SUMMARY

1. The median ocellar nerve preparation of the locust *Locusta migratoria* has been re-investigated.

2. The nerve contains six nerve fibres of which the two largest are 8 μ in diameter.

3. The nerve fibres transmit propagated nerve impulses when the ocellus is illuminated or darkened. This disposes of the claim (Parry, 1947) that the ocellar nerve exerts an effect on the brain as the result of electrotonic spread from a depolarized region in the ocellus.

4. The retinal potentials consist of a reduction of potential at the back of the ocellus on illumination and vice versa on darkening. This is the converse of the situation implicit in Parry's exposition.

5. Three kinds of nervous response are obtained from the ocellar nerve: an 'on'-discharge, an 'off'-discharge and a continuous dark-discharge.

6. Corresponding discharges of nerve impulses are obtained from the circumoesophageal commissure.

7. The ocellus must function principally as an indicator of changes in light intensity.

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URINE PRODUCTION BY THE ANTENNAL GLANDS OF *PALAEMONETES VARIANS* (LEACH)

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INTRODUCTION

The morphology and diversity of the various excretory organs in the invertebrate phyla have frequently been subjects of study, but only rarely have they been investigated from a functional or physiological point of view. This account endeavours to add a little to the present knowledge of excretory organs of the Crustacea and to demonstrate the part played by those organs in osmoregulation. An earlier paper (Parry, 1954) recorded the results of chemical analyses of the excretory fluid of the palaemonid prawn, *Palaemon serratus* (Pennant) when that animal was living in three different salinities. It was established that, although the urine in this species is isotonic with the blood in all conditions of external salinity, the urine serves to remove excess magnesium and sulphate from the body, and this selective excretion is augmented as the external concentration of these ions rises. While the analyses of ions in the blood and urine from animals living in different salinities contributes to our knowledge of the use of the antennal gland, our interpretation of its function as an excretory organ is very incomplete without some estimate of the quantities of salts lost in the excretion. The present inquiry into the volume of urine produced in different conditions by the antennal glands of prawns was undertaken to fill this gap. While *P. serratus* is the best of the available species for chemical analyses of blood and urine since it is the largest, it proved unsuitable for experiments on urine flow since it is very sensitive to handling. The brackish water prawn *Palaemonetes varians* (Leach) is much more amenable to experimental treatment, and was therefore used for this investigation.

The osmotic pressure of the blood and urine in this species under different conditions of salinity were measured by Panikkar (1941). The blood is hypotonic ($\equiv 2{\cdot}3\%$ NaCl) to sea water ($\equiv 3{\cdot}5\%$ NaCl) when the animal is living in that medium. The urine is isotonic with the blood, or very nearly so. Blood and urine are both isotonic with the medium when it is about 60–70% of sea water ($\equiv 2{\cdot}0\%$ NaCl), and at lower salinities both blood and urine maintain their salt concentrations at a level much higher than that of the medium. There is some diminution in the salt content of the body fluids in very dilute media, but even in a medium equivalent to 0·01% NaCl the blood concentration is equivalent to about 1·89% NaCl. In salinities greater than sea water there is similar control of the salt content of the

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body fluids, but it does tend to rise so that in 150% sea water the salt content of the blood is roughly equivalent to 2·5% NaCl. Throughout this salinity range the osmotic pressure and chloride content of the urine show only insignificant differences from those of the blood. The species has a very wide range of tolerance, from water that is nearly fresh to concentrated sea water (≡5·2% NaCl). Through this range of *c.* 5% NaCl, the change in the blood is only *c.* 1·0% NaCl.

The morphology of the excretory organs of palaemonid prawns has been described by Grobben (1880), Weldon (1889, 1891), Marchal (1892), Allen (1892), Cuénot (1895), and Patwardhan (1937). A brief review is given by Panikkar (1941). The excretory organs are antennal glands, except in the larval stages where there is a transitory maxillary gland.

Each antennal gland consists of end-sac, tubular labyrinth, bladder and excretory pore. The end sac is a small compact coelomic sac lying at the base of the antenna. Its wall is considerably folded. There is an outer layer of connective tissue with blood spaces, and an inner convoluted layer which is lined by large epithelial cells which have conspicuous nuclei and a granular cytoplasm. The blood supply to the end-sac has been demonstrated by injections of the blood vessels of live animals. I have dissected injected specimens and reconstructed the arrangement of the blood vessels from serial sections to confirm these observations. The main branch of the antennary artery on either side of the thorax leads directly to the end-sac, where it suddenly splits up into numerous fine vessels which are lost in the walls of the end-sac. Neither the labyrinth nor any other part of the gland appears to have any direct blood supply, although the connective tissue of the labyrinth has numerous blood lacunae, and all the parts of the antennal gland lie within the haemocoele.

The labyrinth is a network of anastomosing tubules which are formed of a single layer of epithelial cells. It leads to the bladder which is lined by a thin pavement epithelium. The bladder communicates with the exterior by a short duct to the excretory pore which opens at the base of the antennary peduncle on a small papilla. The most unusual feature of the antennal gland is the presence of two backwardly projecting arms of the bladders which fuse in development to form a single large 'nephroperitoneal' (Weldon, 1891; Allen, 1892; Patwardhan, 1937; Panikkar, 1941) or 'renal' (Patwardhan, 1937; Panikkar, 1941) sac, lying dorsally in front of the heart and gonad and above the stomach. This structure will be referred to as the 'epigastric' sac. It is lined with the same pavement epithelium as the rest of the bladder; there is no visible histological or structural difference between it and other parts of the bladder. Neither the bladder nor the epigastric sac appears to have any intrinsic muscles, but some muscle fibres seem to run from the exoskeleton of the rostral region to the front of the epigastric sac.

The epigastric sac is overlain by another smaller sac, termed the 'dorsal' sac (Allen, 1892) which has been variously interpreted as a blood space (Weldon, 1889) and as a persistent coelomic space (Allen, 1892). It has no blood corpuscles and no direct communication with any of the blood sinuses of the body (Allen, 1892). It appears to have little significance in the osmoregulation of the animal since Panikkar (1941) measured the osmotic pressure of its contents in *Palaemon serratus* and failed

to find any osmotic difference between it and the fluid of the epigastric sac, or of the excretory pores.

A feature which distinguishes this group from the other decapods which live in fresh water is the absence of a tubule between the labyrinth and the bladder. It is this portion of the excretory system in the crayfishes and gammarids which has become modified to function as a salt-resorbing mechanism in the fresh-water forms (Peters, 1935; Schwabe, 1933). In the palaemonids there appears to be no structure in the excretory organ which could be associated with the accommodation of the animal to different external salinities. Schwabe (1933) was unable to find any difference in the size of the gland in fresh-water or brackish-water forms of *Palaemonetes varians*, in contrast to the variable size of the maxillary gland in gammarid species from different salinities.

Among the Caridiidae there is a good deal of variation in the morphology of the gland, although many of them appear to have some form of epigastric sac, as in *Pandalus*, *Hippolyte*, and *Crangon* (Weldon, 1889). In most cases the labyrinth is reduced. It is almost absent in *Pandalus* and *Hippolyte* and completely absent in *Crangon*. The presence or absence of parts has no apparent bearing on the distribution of these species in different salinities.

MATERIALS

The animals used in the experiments described here were of the species *Palaemonetes varians* (Leach) and were collected either from salt marshes south of the Thames Estuary at Whitstable, Kent, or from salt marshes bordering the River Stour near Manningtree, Essex. Both environments seemed generally to have a salinity about half that of sea water, although this was variable according to the tides, wind and other climatic conditions.

The animals were acclimatized to salinities not very different from that in which they had been living previously, by placing them in the appropriate salinity 3 or 4 days before experiments. In very high or low salinities they were gradually acclimatized for a period of a week, and then kept for a further week in the salinity of the experiment. Measurements of the chloride content of the blood, and osmotic pressure (Panikkar, 1941) indicated that these animals were acclimatized to the particular salinity within the period allowed.

Media with a salinity less than that of sea water were made from Plymouth sea water ($Cl' = 19\,\%$ approximately) and distilled water. For salinities greater than sea water, Plymouth sea water was concentrated by boiling to half its original volume, corrected for pH with drops of sodium carbonate and then diluted with Plymouth sea water to the required salinity. In this way the ionic balance was kept similar to that of ordinary strength sea water. Plymouth sea water is referred to as '100 % sea water' and the other salinities referred to as percentages of this standard.

METHODS AND EXPERIMENTAL RESULTS

In the present investigation several independent techniques have been employed to study the urine flow in *Palaemonetes varians*. Each has certain inherent disadvantages and inaccuracies, but all show the same pattern of urine production in different conditions of external salinity.

(1) *Total excretion of injected dye: a qualitative method*

It was known to many of the earlier investigators that certain non-toxic dyes were excreted by specific organs when introduced into the body of an animal. Previous investigations of crustacean excretory organs by this means were made by Weldon (1889), Marchal (1892) and Lison (1942). Indigo-carmine was chosen as a suitable dye for experiments with *P. varians* from these earlier accounts. When injected in small quantities it appears to be taken up exclusively by the labyrinth and not by the end-sac, as there is always a colourless patch in the region of the end-sac, while the region of the labyrinth becomes deeply stained. From the labyrinth the blue excretory fluid accumulates in the epigastric sac before being lost through the external openings of the glands. The wall of the epigastric sac does not stain with the dye, contrary to the account of Weldon (1889), since it becomes quite colourless when it empties, and then begins to fill again slowly with the blue fluid. The rate at which the stain is removed from the animal appears to be a function of the rate of excretion and this seems to depend on the external salinity. It seems improbable that the dye is changed to a colourless compound in the body since the usual oxidizing and reducing agents fail to alter it between pH 5·5 and 8·5. After the dye is injected it fills the blood spaces and then is gradually removed to the antennal glands. If the animal is moribund and does not excrete, the blue dye remains distributed throughout the haemocoele until the animal dies.

It has been suggested by Palm (1952) that in some insects the rate of excretion of dyes is proportional to their concentrations in the blood so that other factors besides salinity may influence its excretion in prawns.

The experimental procedure was as follows. Animals previously acclimatized to a particular salinity were injected with a small quantity of the stain (*c.* 0·001 ml. of a filtered 1 % solution of the indigo carmine in sea water isotonic with the blood) into the lateral blood sinuses of the abdomen. Larger doses were not so satisfactory, as the dye is then taken up by the cells of the digestive gland which remains so stained for a considerable period. (This phenomenon was observed by Lison, 1942.) This small quantity of fluid injected into the blood alters the blood very little either in composition or in volume, and the animal certainly appears to be quite unaffected by it. Within 5 min. of the injection the epigastric sac begins to appear blue, pale at first, and then increasing in intensity of colour as the sac expands. After it has reached a certain size (which seems to depend on the size of the animal and on the salinity of the medium) the sac empties and the blue-stained excretory fluid is emitted from the excretory pores. When micturition occurs there is a sudden shrinkage of the central portion of the sac, as though a draw-string had been pulled

tight, and it shrinks to two finger-like projections lying on each side of the gut; as the sac fills, these swell and the central part pushes back between them so that it forms a large ovoid sac lying above the stomach. The sac is figured in this state by most authors (Allen, 1892; Weldon, 1889; Patwardhan, 1937; Panikkar, 1941). There is some indication in sectioned material of a group of muscles running from the exoskeleton of the rostrum to the front of the sac. These may assist in its contraction, or emptying may be caused by the action of the thoracic muscles. There are no muscle fibres apparent in the walls of the sac itself.

After injection of indigo-carmine the rate of excretion was measured as the time taken for the complete disappearance of the dye. The end-point of this process was necessarily subjective since there must be some concentration of the dye which cannot be detected, but the manner of excretion by concentrating the dye in a small volume minimizes this error. Some of the variation in the results will be caused by variations in the size of the animal and the size of the dose administered, although both were kept approximately constant. Moulting, sex, and other physiological conditions may also add to variability of the results. In Table 1 the results are expressed as the mean time for the total clearance of the dye from the body and as the mean reciprocals of these times of clearance. The reciprocals are plotted against the external concentration in Fig. 1. Since the volume of the injected fluid was not accurately known, nor the volume of the blood, it was not possible to make a quantitative calculation of the clearance of the dye from the blood.

Table 1. *Time taken for the clearance of indigo-carmine injected into* Palaemonetes varians

Salinity of medium (as % sea water)	Mean time for clearance (hr.)	Mean reciprocal time for clearance ($hr.^{-1}$)	Standard error	Number of animals
5	1·65	0·627	±0·057	5
10	2·00	0·589	±0·046	11
50	4·66	0·234	±0·018	20
70	7·25	0·140	±0·007	8
100	4·36	0·235	±0·014	11
125	2·93	0·398	±0·065	7
150	3·13	0·385	±0·057	11

It appears from these results that the excretion of the dye is slowest when the blood and external medium are isotonic (about 70 % of sea water). It increases as the salinity of the medium drops, but it is also increased somewhat as the salinity of the medium rises. Above 125 % sea water the speed of excretion seems to be halted, since there is no significant difference between the rate of excretion in 125 % and 150 % sea water.

(2) *Excretion of injected dye: a quantitative method*

The injection of indigo-carmine into prawns, described in the previous section, enables the excretory organ, especially the epigastric sac, to be observed during the excretion of the dye. This has been used as a further method of determining the

urine production in different salinities and of making some quantitative estimate of the urine flow.

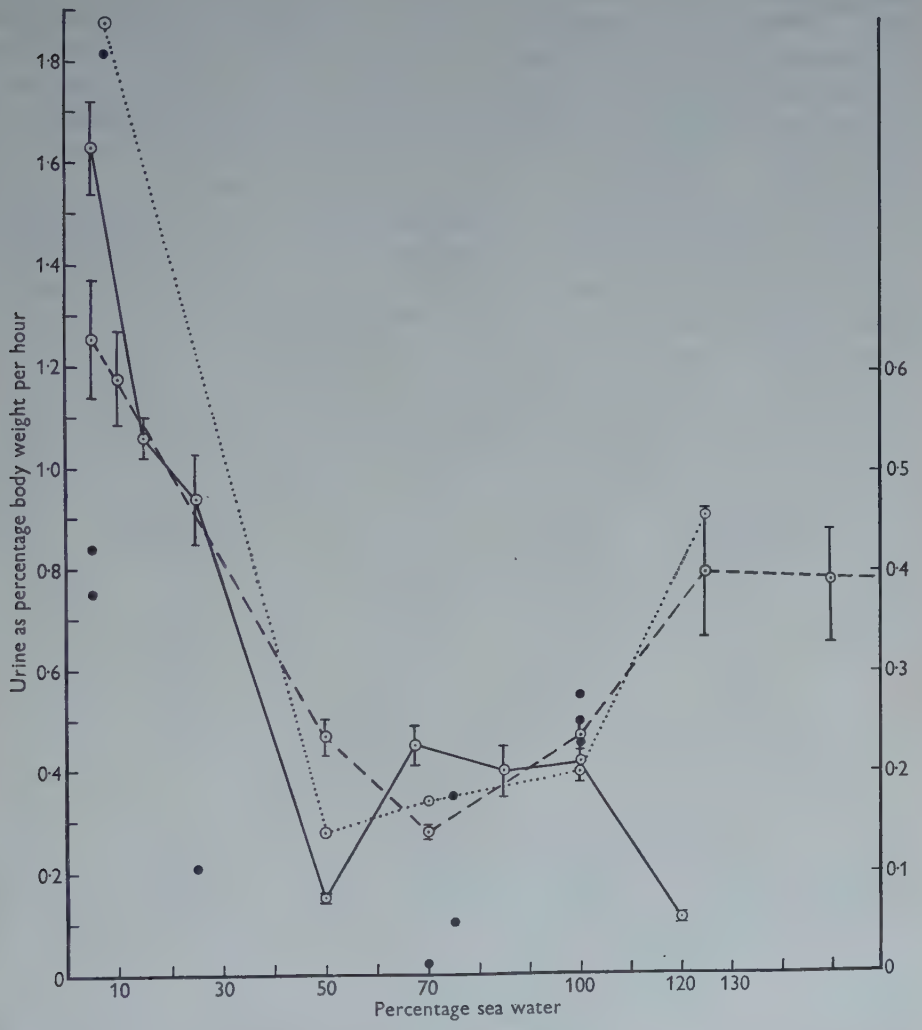

Fig. 1. ⊙—⊙, urine flow estimated from size of epigastric sac; ⊙– –⊙, urine flow estimated from clearance of dye (ordinates are reciprocals of excretion times on the right-hand scale); ⊙. . .⊙, urine flow estimated from weight changes after blocking pores; ●●, urine flow estimated from cannulation of one excretory pore. Ordinate: urine expressed as percentage of body weight excreted in 1 hr. Abscissa; Salinity of medium expressed as a percentage of Plymouth sea water.

The approximate volume of urine produced was measured by estimating the maximum size attained by the epigastric sac and the times of micturition. The size of the sac was measured with a squared eyepiece and the measurements converted to cubic millimetres. The sac was observed continually, and the length and breadth of the sac was measured every few minutes until micturition occurred. The volume

of urine excreted in a certain time was estimated and expressed in terms of body weight. The figure used for 'volume' was obtained as the product of length, breadth and depth of the epigastric sac, and so will give a somewhat higher value than the true one since its shape is that of an ovoid sac and not a rectangle. As this shape was constant in all animals the estimated volumes are comparable with each other and proportional to the true volume. The animals were weighed immediately after each experiment. They all appeared healthy and survived handling in the experiments without adverse effects.

The estimated urine production in several different salinities is given in Table 2 and plotted against the salinity of the medium in Fig. 1. There appears to be a minimal flow of urine in a medium of about 50% sea water and a rapid rise in the volume excreted as the salinity of the medium is lowered. In the more saline media the flow appears to increase somewhat and is thereafter approximately constant in a medium between 70 and 100% sea water.

Table 2. *Rate of urine flow in* Palaemonetes varians: *measurement of volume of epigastric sac after injection of indigo-carmine*

Salinity of medium (% sea water)	Urine as % body weight per hour	Standard error	No. of experiments
5	1·63	±0·09	34
15	1·06	±0·04	10
25	0·94	±0·09	25
50	0·15	±0·01	10
67	0·45	±0·04	14
85	0·40	±0·05	13
100	0·42	±0·04	12
120	0·11	±0·01	6

(3) *Weight changes after excretory blockage*

The third method of investigation was the classical one of stopping the excretory organs and measuring the subsequent weight changes. This method was used for comparison with the published data of excretion in other decapods. The most satisfactory material for blocking the pores was found to be dental cement, which was mixed freshly to a thin suspension and then pipetted into the opening. If the cement is used to cover the surface of the excretory pore, the mouthparts remove it very quickly. The method is unsatisfactory in that it is impossible to be sure that the pores are adequately blocked until the expected changes begin to take place. The method assumes that the changes in weight are all due to the accumulation of unexcreted urine, but there may be other explanations. The increase in weight could be produced by swallowed water. Although swallowing has been observed by adding nigrosin to the medium and by some previous authors (Panikkar, 1941; Fox, 1952) quantitative and consistent observations could not be made to check this.

The animals were weighed immediately after stopping the openings of the glands, and thereafter at hourly intervals. The results were variable, although

each animal showed a steady trend of gain or loss in weight. In Table 3, the mean percentage gain in weight per hour is given. Measurements were made every hour for a period of up to 8 hr., but the results were calculated from the first 4 hr. The column of figures marked 'E.P.O.' in the table represents animals with open excretory pores; these are the controls of the experiments. From 5 to 100% sea water there are only very insignificant changes in the weight of these control animals. In these experiments some of the control animals were found to gain, some to lose a little weight, perhaps due to defaecation or drinking. The column of figures marked 'E.P.Bl.' refers to animals with sealed excretory pores. Although animals in media up to 125% sea water will survive for at least 24 hr. with blocked excretory pores, in higher salinities they do not survive well and no satisfactory measurements could be made on animals living in media more concentrated than 125% sea water.

Table 3. *Rate of urine flow in* Palaemonetes varians: *measured by weight changes after blocking the excretory pores*

Salinity of medium (% sea water)	Mean percentage increase in weight per hour				Urine as % body weight per hour
	E.P.O.		E.P.Bl.		
	Range	Mean	Range	Mean	
0	1·12– 2·23	1·68 (6)	2·00–2·65	2·35 (3)	—
5–10	−0·06– 0·06	0·02 (6)	0·60–2·80	1·89 (8)	1·87
50	−0·03– 0·06	0·03 (3)	0·28–0·84	0·31 (3)	0·28
70	−0·11– 0·17	0·03 (4)	0·08–1·05	0·37 (9)	0·34
100	−0·29– 0·37	−0·10 (5)	0·20–0·41	0·30 (4)	0·40
125	−0·12––0·50	−0·27 (3)	0·29–1·61	0·64 (8)	0·91
150	−1·22––1·85	−1·43 (3)	—	—	—
200	−3·84––4·26	−4·08 (3)	—	—	—

The number of measurements upon which the mean is based is given by the figure in parentheses.

The rate of urine production has been calculated from the two sets of observations in the columns E.P.O. and E.P.Bl. For the range 5–70% sea water the urine produced is taken as the difference between the E.P.O. and the E.P.Bl. figures. In the hypertonic solutions the 'open' animals lose weight presumably as a result of an osmotic outflow of water, so that the E.P.Bl. figure represents the weight of urine produced, less the osmotic water loss. The sum of E.P.O. and E.P.Bl. is taken to represent the weight of urine in such circumstances. The urine production is calculated only for the range 5–125% sea water as outside this range there is only limited survival after blocking the excretory organs. The E.P.O. figures above and below these salinities show that there must be a considerable osmotic flux of water in markedly hypo- or hypertonic conditions.

It appears from these results that there is little difference to be observed in the quantity of urine produced between 50 and 100% sea water, but this may be a reflexion of the inadequacy of the method. An increased rate of urine production in hypo- and hypertonic media is indicated by this method.

(4) *Direct measurement of urine production by cannulation*

The final method to be described was a more direct one than any of the previous ones and consisted of cannulating one of the excretory pores. Although its directness has advantages, it was difficult to execute, and many possible errors were involved. Cannulation of the excretory pore may lead to stimulation of micturition, and the amount of urine present in the epigastric sac at the beginning and end of each experiment cannot be taken into account. As no measures were taken to prevent the escape of urine from the 'open' excretory pore, the volume collected from the one was doubled to account for both. These sources of error made it unfruitful to continue these experiments which serve only to amplify the evidence provided by other means. Some collections of urine were made from *Palaemon elegans* (Rathke) (=*Leander squilla* (L.)) and *P. longirostris* (Milne-Edwards), which are included here for comparison.

Table 4. *Rate of urine flow in* Palaemonetes varians: *measured by cannulating one excretory opening*

Salinity of medium (% sea water)	Species	Urine as % body weight per hour
5	*P. varians*	0·75
5	*P. varians*	0·84
25	*P. varians*	0·21
70	*P. varians*	0·02
100	*P. varians*	0·50
100	*P. varians*	0·46
100	*P. varians*	0·55
7	*P. longirostris*	1·82
75	*P. elegans*	0·10
75	*P. elegans*	0·35

The figures in the last column are calculated to include both glands.

The animal was laid on its back on a bed of cotton-wool between two banks of sealing wax, fastened down with cotton threads and wax, and then placed in a bath of the appropriate salinity (to which it had been acclimatized) beneath a binocular microscope. The cannulae used were of ordinary soda-glass, drawn to a steep taper, with the fine tip softened in a micro-flame so that it should have no jagged edges. The cannula was held in a small clamp. By the application of a slight pressure the fluid in the bath was prevented from rising by capillarity while the cannula was arranged. The presence of the cannula did not appear to harm the animal in any way, as most animals survived the experiment indefinitely. In many cases the experiment failed because the cannula became blocked or because the animal moved and displaced the cannula, so allowing the external medium to leak into it. The fluid collected was tested for osmotic pressure or chloride, since there should always be some concentration difference between it and the medium, except in those media in which blood, urine and medium are isotonic. The expected concentration of the urine could be calculated from Panikkar's (1941) osmotic pressure data, or from chloride analyses of the blood.

The results obtained by this cannulation method are given in Table 4, the urine flow being expressed as the percentage body weight excreted per hour. Although the results are few in number, they do confirm those of the other three methods, namely that the urine flow is high in hypotonic media (as in brackish water *c.* 5 % sea water), minimal in isotonic media and high again in a hypertonic media. The urine flow measured by this method is similar in magnitude to that estimated by the other quantitative methods.

DISCUSSION

The general pattern of excretion shown by these diverse methods indicates a slow flow of urine in media isotonic, or nearly isotonic, with the blood. If the medium becomes hypotonic the urine flow is increased—a tenfold decrease in the salinity of the medium apparently inducing a tenfold increase in the volume of urine produced. Thus, in 50 % sea water, the urine flow has been estimated as 0·15 % body weight per hour; while in 5 % sea water it has risen to 1·63 % body weight per hour (from the observations of dye excretion, method 2). In changing the medium from 50 to 5 % sea water, the flow of urine appears to have increased progressively. In media more concentrated than isotonic there is a tendency for the urine flow again to be increased, although the rise is a comparatively small one and after the minimal flow has been approximately doubled, it appears to be kept at a relatively steady level while the medium continues to increase in salinity (again using the observations of the second dye excretion method).

The salinity of the medium at which the urine flow is minimal is variable between samples of animals and may reflect differences in the method of estimating urine flow, or it may reflect different physiological states, different races acclimatized to different habitats, or the different times of the year when the experiments were made. The minimal urine production in the second set of experiments appears at a lower salinity than in the previous dye-injection method. This may be associated with the different environments in which the animals were found (the first were from salt marshes at Whitstable, Kent; the second from salt marshes near the River Stour, Essex), or perhaps with the different times of the year when the experiments were made (the first during May-June, 1953; the second during March-April, 1954). This shift in the minimum might thus be attributed to a seasonal change in the animals (such as the drop in osmotic pressure of the blood in summer recorded by Panikkar, 1941) or to the results of a long-term acclimatization to different environments. In spite of these differences, however, the general pattern of excretion is so similar in the different methods employed that some conclusions may be based upon this estimate of urine production.

It is difficult to compare these results with those recorded for other decapods (Table 5) since, with the exception of the figures for *Eriocheir*, all the previous records refer to fresh-water animals (such as *Cambarus* and *Potamobius*) or to marine animals (such as *Maia*, *Cancer* and *Carcinides*). Only for *Carcinides* are there experiments indicating a varying flow following a change in the salinity of the medium. Nagel's results (1934) indicate that the urine flow is roughly doubled

when the salinity of the medium is reduced from 100 to 50 % sea water—a gradient of change in the urine flow which appears very similar to that found in the present investigation for *Palaemonetes varians*, when transferred from an isotonic medium to dilute brackish water (from about 50 % sea water to 5 % sea water).

Table 5. *Rate of urine flow in some decapod Crustacea*

Species	Medium	Author	Urine as % body weight per hour
Maia	Sea water	Bialaszewicz (1932)	0·125
Cancer	Sea water	Robertson (1939)	0·125–0·416
Eriocheir	Fresh water	Scholles (1933)	0·175
Carcinides	Sea water	Nagel (1934)	0·416
Carcinides	$\frac{2}{3}$ sea water	Bethe *et al.* (1935)	0·300
Carcinides	$\frac{1}{2}$ sea water	Nagel (1934)	0·708
Cambarus	Fresh water	Lienemann (1938)	0·217
Potamobius	Fresh water	Herrmann (1931)	0·158

In general the figures for the urine flow in *Palaemonetes varians* in the middle range of its habitat (25–100 % sea water) are of similar magnitude to those recorded for other marine decapods which are approximately isotonic with the sea water in which they live. *Carcinides* in 50 % sea water has a blood concentration considerably higher than that of the medium, and its urine flow is apparently much faster than when it is in an isotonic medium. There are no estimates of urine production in lower salinities, and indeed they would be difficult to make since *Carcinides* will not live successfully in salinities lower than this. We may perhaps assume that, as the salinity is lowered, the urine flow of such an animal will increase, as it appears to do in *Palaemonetes*. But the fresh-water Crustacea, and even *Eriocheir* which may be regarded as a successful marine invader of fresh water, have generally a much lower urine flow, in contrast to the greatly augmented flow in *Palaemonetes* in water which is nearly fresh. The urine flow of this prawn in 5 % sea water is nearly 10 times as great as that recorded for *Eriocheir* in fresh water. If the urine flow of *Palaemonetes* acclimatized to even lower salinities were measured, it seems probable that the flow would be even greater than that recorded for 5 % sea water. This leads one to expect that in *Eriocheir* in fresh water some further means of osmotic control has been brought into play, which perhaps reduces the salt loss through the antennal glands or reduces the osmotic influx of water.

Whether some other mechanism is present in the fresh-water variety of *Palaemonetes* we do not know, but the evidence suggests that this must be so. The fresh-water variety of *Palaemonetes* has been accorded racial status as *P. varians* var. *macrogenitor* on the basis of morphological studies (Sollaud, 1923, 1932), and the only record of the osmotic pressure of the blood of the fresh-water variety (Vialli, 1925) indicates that this may be very much lower ($\Delta = 0{\cdot}54°$ C.) than that recorded for *P. varians* from Britain ($\Delta = 1{\cdot}28$–$1{\cdot}40°$ C.) (Panikkar, 1941). The lowest value recorded by Panikkar for animals gradually acclimatized to almost fresh water (0·01 % NaCl $\equiv \Delta$ 0·006° C.) was for blood, 1·982 % NaCl $\equiv \Delta 1{\cdot}18°$ C. The methods

used for measuring osmotic pressure were very different (Vialli used Monti's thermo-electric method; Panikkar a Hill-Baldes thermocouple; I have confirmed Panikkar's figures using Ramsay's freezing-point apparatus (1949)) but even allowing for some degree of error in Vialli's results there still seems to be a significant difference between these figures.

This possible difference between two varieties of *P. varians* is interesting in the light of the energy required for osmotic work in such conditions. A marine *Eriocheir* put into fresh water would use about 11 % of its total available energy for osmotic work (Potts, 1954), but a specimen of *Eriocheir* adapted to fresh water only uses 0·54 % of its total energy since the blood concentration is considerably reduced. The urine in a fresh-water *Eriocheir* remains isotonic with the blood after transference from sea to fresh water, but if it were of lower concentration than the blood, a further saving of energy would be achieved. Truly fresh-water Crustacea, such as the crayfishes, have a urine which is very dilute, thus using a minimum of energy for the maintenance of the osmotic difference between internal and external media.

It is clear from this that the variety of *Palaemonetes varians* found in Britain could only extend into fresh water at considerable energetic expense, if the blood and urine concentrations are to be maintained at the level shown by animals in 0·01 % NaCl (the lowest medium for which Panikkar (1941) recorded a determination of the osmotic pressure of the blood). On the other hand, the fresh-water variety of *P. varians* from southern Europe appears to have a very low osmotic pressure of the blood, so that even if the urine is still isotonic with the blood, the animal will be saving a considerable amount of energy in comparison with a brackish water specimen in a very dilute medium. *P. varians* seems never to have been recorded from completely fresh water in Britain, and indeed seems restricted to certain rivers of southern Europe (Boas, 1898). The geographical separation and the considerations of the energy necessary to maintain the brackish water variety in fresh water seems to support the concept of two distinct races for this species, previously based upon morphological and embryological observations. The fresh-water variety of *P. varians* should thus provide an interesting study in osmoregulation with some of the modern methods of investigation.

In the brackish water variety of *P. varians* with which this study is concerned, the osmotic work done by the animal is dependent on the permeability of the integument to the various constituents of the medium. From the results of the experiments described in this paper, and from some recorded briefly in the Appendix (p. 420), certain facts are established. First, it is clear from the experiments involving weight changes of animals with open excretory pores in distilled water and in media more concentrated than 125 % sea water, that water is gained or lost with facility in markedly hypo- or hypertonic media. Secondly, experiments with heavy water (see Appendix, p. 420) suggest a rapid exchange of water in hypo- (5 % sea water), iso- (70 % sea water) and hypertonic (120 % sea water) salinities. The half-time of penetration of heavy water in these salinities was between 0·43 hr. and 0·73 hr. Thirdly, experiments with an isotope of sodium, ^{24}Na (see Appendix, p. 421), suggest a rapid exchange of sodium in different salinities. The half-time of exchange (outflow) was

about 2–3 hr. in 5 % sea water, 1–1½ hr. in 70 % sea water and 1½–2 hr. in 120 % sea water.

From these conclusions we must assume that in the salinities in which the animal normally regulates without prolonged acclimatization, i.e. from 1 % sea water to 120 % sea water, there must be some mechanism compensating for these influxes and outfluxes of water and ions. The production of urine cannot be considered instrumental in maintaining the 'steady state' of the animal. While the progressively increased flow of urine in hypotonic media may reflect and counteract the inward osmotic flow of water, much essential salt is lost at the same time. In hypertonic media, the apparent increase in the flow of urine loses water from the animals as well as salts, and this loss must be made good elsewhere.

SUMMARY

1. Four methods for estimating the rate of urine flow in *Palaemonetes varians* are described.

2. The rate is minimal when the external medium is approximately isotonic with the blood. All methods indicate that the rate increases progressively with increasing dilution of the external medium below 50 % sea water. There is some evidence to suggest that the rate increases in hypertonic external media.

3. These results are discussed in relation to estimates of the urine production in some other Crustacea and in relation to the ecology of the genus *Palaemonetes*.

I should like to thank Prof. Sir James Gray, F.R.S., and Prof. H. Munro Fox, F.R.S., for the facilities offered by their departments.

APPENDIX

Experiments to determine the exchange rates of water and sodium ions were planned using D_2O and ^{24}Na. These experiments did not provide sufficiently precise data for a calculation of the exchange rates, but did demonstrate the permeability of *Palaemonetes varians* to heavy water and the sodium isotope. A brief summary of these experiments is appended.

(1) *Heavy-water experiments*

Animals were placed in solutions of heavy water (*c.* 20 %) and different salinities (5, 70 and 120 % sea water) and the rate of penetration of the heavy water measured. This was done by taking blood from the animals at half-hourly intervals, distilling the water from this sample, and estimating its heavy water content by measuring the density of the distillate (using a simple modification of the method of Fenger-Eriksen, Krogh & Ussing, 1936).

The mean half-time of penetration in the three salinities was as follows:

5 % sea water, $t_{\frac{1}{2}} = 0{\cdot}53 \pm 0{\cdot}14$ hr. ($n = 12$),

70 % sea water, $t_{\frac{1}{2}} = 0{\cdot}73 \pm 0{\cdot}28$ hr. ($n = 8$),

120 % sea water, $t_{\frac{1}{2}} = 0{\cdot}43 \pm 0{\cdot}15$ hr. ($n = 8$).

(2) *Sodium isotope experiments*

Animals were left in a solution of ^{24}Na and various salinities (5, 70 and 120% sea water) for 24 hr. After this time the radioactive count of the animal did not rise appreciably, and it was assumed that all the unbound sodium had been exchanged. The animal was then placed in front of the window of a GM 4 tube and washed in a constant current (3 ml./min.) of the non-active medium. The radioactivity of the animal was counted at half-hourly intervals until it was reduced to an insignificant level. Some typical measurements of the half-time of washing-out were as follows:

Medium	Half-time (hr.)
5% sea water	3·25
5% sea water	2·30
70% sea water	1·50
70% sea water	1·40
70% sea water	0·85
120% sea water	1·65
120% sea water	1·90

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OSMOREGULATION AND IONIC REGULATION IN *HYDRA*

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INTRODUCTION

So far as is known all fresh-water animals have a mechanism enabling them to maintain their cells and body fluids hypertonic to the external medium, despite the continuous osmotic inflow taking place across their body surfaces. Frequently this mechanism is in the form of a kidney removing excess water but resorbing salts. In addition to this, there is often an active uptake of salts occurring either through the general body surface, as in the case of frog skin (Krogh, 1937), or by special groups of cells such as the anal papillae of Diptera (Koch, 1938). The task of osmoregulation is no doubt made easier by restriction of the permeable area of the body surface, as in Crustacea.

In the fresh-water Coelenterates, no excretory structure has been observed, and there is no information as to how the internal osmotic concentration is maintained. There are four possibilities to be considered: (1) The body fluid could be isotonic with the external medium. However, reference to all other fresh-water animals investigated suggests that this is unlikely. (2) An impermeable body surface would prevent any osmotic inflow. Although some fresh-water animals have an integument which is, in general, impermeable to water and salts, nevertheless some small part of the body surface is of a permeable nature. It therefore seems unlikely that fresh-water Coelenterates will be found to have a completely impermeable body surface. (3) The cell surface, although semi-permeable, could be strengthened in such a manner as to enable it to withstand the considerable hydrostatic pressure which would be brought upon it by any serious difference in the concentration of the solutions on the two sides. The cell membrane of the fresh-water Coelenterate *Hydra* does not show any structure that could be considered to provide support against such a pressure, and even were the cell membrane sufficiently strengthened, the turgid condition of the cells would make any movement of the animal very difficult. (4) The final possibility is that the animal has a selectively permeable membrane and also some active osmoregulatory mechanism. Such a mechanism would either remove the water diffusing into the cells from the external medium or in some way actively oppose the entry of water, as has been suggested to occur in *Procerodes ulvae* (Beadle, 1934). In addition, a means of salt uptake from the external medium would probably be needed for the replacement of salts leaking out.

For an investigation of the osmoregulation of fresh-water Coelenterates such as *Hydra*, a knowledge is clearly needed of the internal osmotic concentration of the

cells and of the permeability of their surfaces to water and various solutes. The work to be described in this paper has therefore been divided into two sections: first, a section dealing with the permeability of *Hydra* to water, and, secondly, a section concerning the exchange and uptake of certain common ions, such as Na^+, K^+ and Br^+, under controlled conditions. A knowledge of the rate of entry of ions, and of the internal levels maintained at a steady state, is necessary for the understanding of the cellular mechanism concerned.

MATERIAL

The fresh-water Coelenterates *Hydra viridis* and *Pelmatohydra oligactis* have been used for this work.

The cultures of *Hydra* were fed on *Daphnia* or *Artemia* at frequent intervals and the discarded carcasses were removed from the culture the day after each meal. The cultures were thoroughly cleaned every fortnight, and the water replaced by fresh pond water of approximately the same pH (7·0–7·6). Failure to clean the cultures leads to a fouling of the water, and the *Hydra* go into a state of depression, as described by Galtsoff *et al.* (1936), and finally die.

PERMEABILITY TO WATER

Method

Experiments were carried out on *Hydra viridis* to determine to what extent its surfaces are permeable to water. *Pelmatohydra oligactis* was not used for these experiments because it is not so easy to distinguish between ectoderm, endoderm and enteron in the living animal of this species.

The experiments were carried out both on isolated tentacles and on whole organisms. Single tentacles were cut off by means of a fine needle. They were placed in freshly aerated water and kept at a constant temperature for 3–4 hr.; these conditions are necessary for successful and healthy regeneration of *Hydra* fragments (Weimer, 1934). During this time the cut ends fused together, so forming a closed tube of ectoderm and endoderm surrounding the enteron. No further reconstruction occurred, because no hypostome was present (Tripp, 1928; Dobrochotow, 1940). As a test of the health of the stock used, pieces of the body of the experimental animals were also kept under similar conditions, and the subsequent regeneration of these pieces observed until they formed a hypostome and tentacles.

Isolated tentacles, which had sealed up as described above, were mounted in a cavity slide on the stage of a microscope, and were irrigated continuously during the experiments. Except in certain earlier experiments the irrigating fluid was kept at a constant temperature ($\pm 0{\cdot}5^\circ$ C.) by being passed through a water-jacket cooled by carbon dioxide snow. Many earlier experiments carried out at temperatures above 20° C. were spoilt by the unhealthy condition of the tentacles. In all experiments above 20° C. the tentacles underwent sudden shortenings and rounded up, and this occurred even in about 25 % of the experiments carried out below 20° C. In these cases there was a curious increase in the combined volume of ectoderm and meso-

gloea, as though fluid had been forced into the latter. Experiments in which such shortenings occurred were discarded on the grounds that the tentacles were not healthy.

The tentacles were irrigated first with the pond water used for culturing, then with a known concentration of sucrose in pond water, and finally in some cases with pond water again. During the experiments photographs were taken at suitable intervals with a Leica 35 mm. camera. In this way a permanent record was obtained of any shrinking or swelling of the tentacles. The volume of ectoderm (with mesogloea), and of the endoderm, was calculated from figures obtained by traversing the photographs with a first moment integrator. It is not possible to separate ectoderm and mesogloea in the photographs.

A moment integrator can be used to measure volume only when the object is symmetrical about an axis of rotation. The appearance of isolated *Hydra* tentacles suggests that they have rotational symmetry, but tests were carried out to verify this. The tentacles were marked on one side with carbon particles and photographs were taken at different angles of rotation. The result obtained at the different positions ranged within $\pm 10\%$ of the mean.

An obvious criticism of the experiments on isolated tentacles is that they are carried out on unnatural and possibly unhealthy preparations. A second set of experiments on a small number of whole *Hydra* was therefore carried out to verify the results obtained on isolated tentacles. There are, however, two disadvantages of using whole animals. First, the whole animal is not symmetrical about a central axis, the hypostome being irregular. This difficulty was overcome by using animals with a bud which served as a mark; only changes in volume below the bud were measured. Secondly, the endoderm of the body is not sufficiently distinct to be measured accurately, and so only measurements of ectoderm were obtained.

Results

(1) *Control experiments*

The results of control experiments, in which the tentacle was photographed in different states of shortening or lengthening, are given in Table 1. These results show that within the limits of the inevitably large experimental error the volume of ectoderm and endoderm does not vary with shortening and lengthening of the tentacle. A significant change in enteron volume, however, was found in two of the five experiments.

Table 1

Exp. no.	Duration of exp. in min.	No. of photographs	Maximum deviation from mean (%)		
			Ectoderm	Endoderm	Enteron
16	70	5	3·5	10·0	8·1
17	57	10	7·1	6·9	22·8
21	$12\frac{1}{2}$	5	3·0	4·0	19·0
23	$17\frac{1}{2}$	5	2·0	4·6	4·3
24	30	6	5·6	9·2	10·0

(2) *Experiments with sucrose*

The table of results (Table 2), together with the graphs (Figs. 1, 2), show that there was a decrease in the volume of ectoderm, endoderm and enteron in concentrations of sucrose greater than 0·04 M.

Table 2

Exp. no.	Concentration of sucrose	Length of exp. (min.)	Temp. (° C.)	% vol. change		
				Ectoderm	Endoderm	Enteron
76	0·20M	27½	12·0	−56*	− 9*	−100*
83		44½	19·5	−50*	−14*	−100*
100		48½	13·0	−53*	−26*	−100*
105		56	19·0	−51*	−25*	−100*
126		25 followed by 1¼ hr. return to pond water	15·5	−20	− 3	− 6
48	0·10M	33	17·0	−25	− 7	− 50
49		30	17·0	−22	− 6	− 46
109		49½	16·5	−28	−11	− 16
113		48½	16·0	−32	−11	− 16
125		20 followed by 3 hr. return to pond water	18·5	−30	0	− 4
43	0·05M	16¼	17·0	−12	− 9	− 20
73		41	17·8	−12	−10	− 20
73		39	19·5	− 2	+ 8	− 20
115		50¼ followed by 13¼ hr. return to pond water	15·0	−16	− 6	− 24
124		62¼	12·0	−13	−11	− 57
81	0·04M	20	21·5	0	− 2	+ 2
91		69 followed by 27 min. return to pond water	?	1·0	− 3	+ 15
119		30½ followed by 26 min. return to pond water	15·0	0	0	+ 2
123		90 followed by 1½ hr. return to pond water	13·0	0	0	0

* Figures indicate that the experiment was continued sufficiently long for a steady volume to be reached whilst in the sucrose solution.

At a concentration of 0·2 M sucrose the enteron soon became obliterated and the ectoderm decreased by approximately 52 %, reaching a steady value almost immediately after the disappearance of the enteron. The figures for endoderm volume are subject to error due to the difficulty of determining the border between endoderm and enteron, but the volume changes recorded do indicate a steady decrease in volume, which is smaller than that occurring in the ectoderm. In general, tentacles which had been for any length of time in a solution of sucrose stronger than 0·04 M did not recover on return to pond water. After short periods of treatment the ectoderm swelled again, on return to pond water, but the enteron remained shrunk in most cases, whether the treatment had been long or short.

Results of six experiments on whole organisms (two in 0·2 M sucrose, one in 0·10 M sucrose, two in 0·05 M sucrose, and one in 0·04 M sucrose) show similar shrinkages of the ectoderm to those obtained on isolated tentacles. As already stated, the endoderm could not be measured.

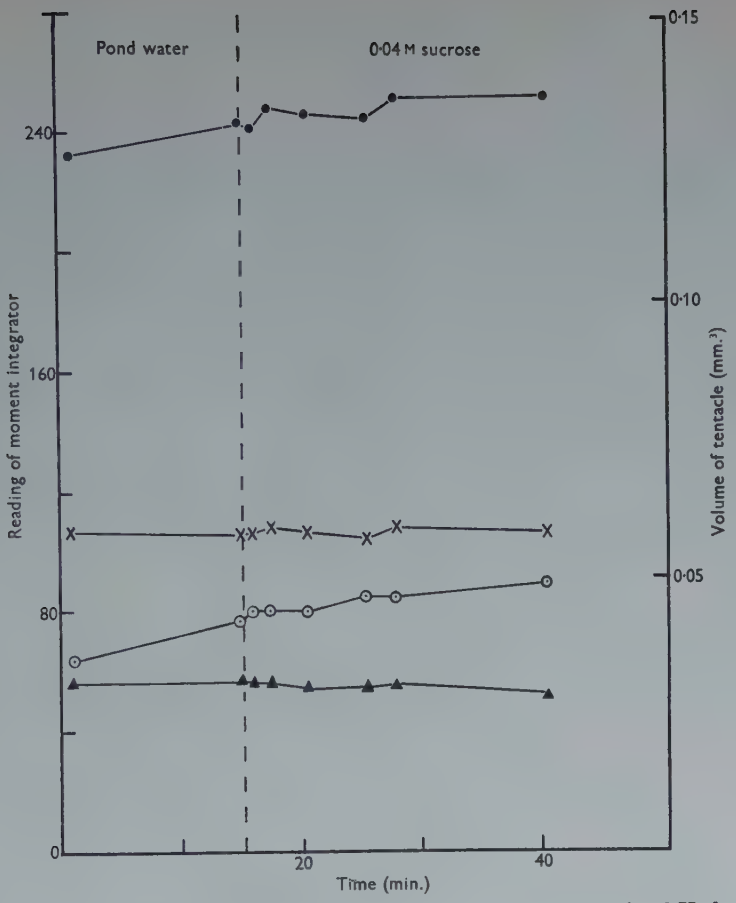

Fig. 1. Effect of a 0·04 M solution of sucrose on the volume of a tentacle of *Hydra viridis*. ●, whole tentacle; ⊙, enteron; ×, ectoderm; ▲, endoderm.

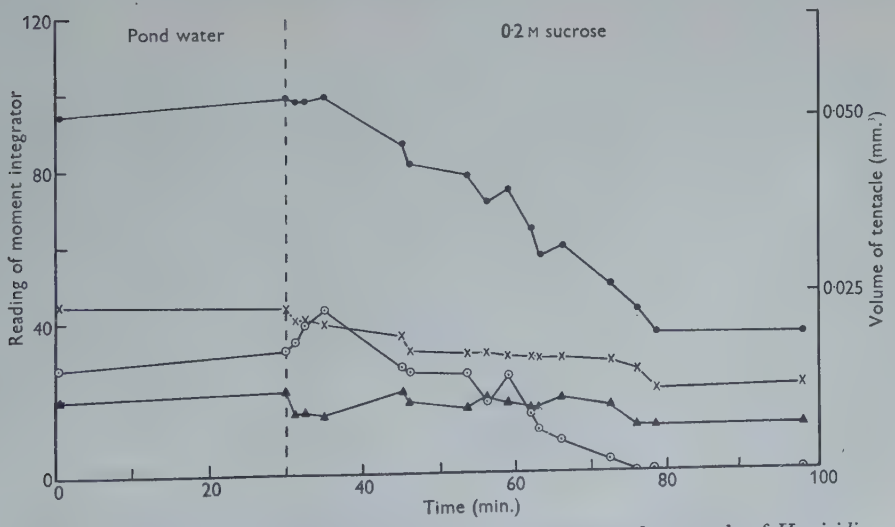

Fig. 2. Effect of a 0·20 M solution of sucrose on the volume of a tentacle of *H. viridis*. ●, whole tentacle; ⊙, enteron; ×, ectoderm; ▲, endoderm.

Conclusions

(1) *Internal osmotic pressure*

Shrinkages of ectoderm and endoderm of isolated tentacles, and of ectoderm of whole animals (endoderm not being measured in these) were only detectable in solutions of sucrose more concentrated than 0·04 M. Irregular fluctuations in the volume of the enteron, associated with changes of shape and possibly due to leaking at the 'seal', occurred even in some control experiments, but a steady decrease was found only with solutions of sucrose more concentrated than 0·04 M. These facts can most readily be interpreted by the assumption that the internal osmotic pressure of the *Hydra* cells is approximately that of a 0·04–0·05 M solution of a non-electrolyte. However, this estimate may be vitiated owing to the presence of an osmoregulatory mechanism. Some additional evidence might be obtained by measurements with the Hill vapour-pressure technique (1930), or the Ramsay freezing-point technique (1949), although the cytolysis necessary for the application of these methods might cause a change of cellular osmotic pressure.

(2) *Osmotically inactive volume*

In the experiments with 0·2 M sucrose the volume of the enteron decreased to zero, and at or shortly after this time the ectoderm and endoderm (within the limits of experimental error) attained steady volumes averaging 48 and 79 % respectively of the original. If the original internal osmotic pressure was equivalent to 0·04 M sucrose, and if sucrose did not penetrate, then the volume of osmotically inactive material in the ectoderm and endoderm may be calculated as 40 and 74 % respectively. These estimates are very approximate and dependent on assumptions which may not be fully justified. The high value for endoderm may be due to stored food material present. Greenwood (1888) describes fat globules and protein material present as stored food in the endoderm cells.

(3) *Permeability to water*

The nature of the barriers through which water has to pass to enter the cells of *Hydra* is not known, nor is it known to what extent the mesogloea is physiologically isolated from the external medium. However, it is very clear from the results that water can pass readily through the tissues of the tentacle. An approximate calculation of the permeability of the tentacle wall comprising ectoderm and endoderm has been made from the formula given by Lucké & McCutcheon (1932, p. 104, equation 5). The values obtained from the results of three experiments with 0·1 M sucrose and three experiments with 0·2 M sucrose were 0·56, 0·48, 0·54 μ^3/μ^2/atm./min. and 0·79, 0·78 and 0·47 μ^3/μ^2/atm./min. respectively. If the water were to pass through four similar cell membranes in series then the permeability of any one of these would have to be 4 times the value given; this seems rather a high figure. But it seems quite possible that much of the water may pass through intercellular spaces.

However, the experiments have also shown that the combined ectoderm and mesogloea shrink considerably under osmotic influences. Although it has not been

possible to distinguish ectoderm from mesogloea in the living material, it may be shown that a large part of the shrinkage observed must have taken place in the ectoderm. Sections of *Hydra* fixed by conventional methods show the mesogloea as a thin line, but sections of material treated by the Altmann Gersh freeze-drying technique show a distinct band traversed by fibrils between the ectoderm and endoderm. The thickness of the mesogloea is not known, but on the basis of the frozen-dried sections the volume is here estimated as about 12 % of the ectoderm and mesogloea combined. Thus the shrinkage of over 50 % found in the ectoderm and mesogloea must be attributed mainly to the ectoderm. Further evidence for this is supplied by frozen-dried sections of animals which had been put into 0·2 M sucrose for 20 min. In these sections, although ectoderm and endoderm cells have a shrunken appearance and broken cell membranes, the mesogloea still occupies approximately the same volume as in normal sections.

By using the formula derived by Lucké & McCutcheon (1932, p. 103, equation 3*a*), and making allowance for osmotically inactive material and ignoring the inner surface of the cells, values of 0·25–0·28 μ^3/μ^2/atm./min. have been obtained for permeability of the outer ectodermal surface. However, while water is being withdrawn osmotically from the ectoderm to the outside medium, it is probably at the same time entering the ectoderm through its inner surface from the enteron. The figures obtained are therefore only minimum values.

EXCHANGE AND UPTAKE OF IONS

Method

In *Hydra* the small quantities involved in the uptake and exchange of ions makes these processes difficult to study by the usual chemical methods. However, by the use of radioactive isotopes it is possible to obtain clear results, as this method is sufficiently sensitive to reveal the small changes of concentration occurring in a single individual.

For all experiments on ionic uptake *Pelmatohydra oligactis* was used. This was because the reproductive rate of this species is greater than that of *Hydra viridis*, so making it more suitable for experiments where large numbers of animals are used; and also because their larger size eased certain technical difficulties, such as washing and weighing.

The stock *Pelmatohydra* cultures were kept in pond water of a pH of 7·0–7·6, and experimental animals were transferred a few days before the start of each experiment to a solution containing NaCl, KCl, $CaCl_2$, $NaHCO_3$, and K_2HPO_4; the relative concentrations of the salts were adjusted for each experiment on the basis of results obtained from previous experiments on the viability of *Pelmatohydra*. These were carried out as follows: some *Pelmatohydra* were placed in a series of solutions (ten in each solution) and their length of life recorded. The solution best suited both to the healthy condition of the *Pelmatohydra* and to the requirements of the experiment was then selected.

Feeding of animals used in each experiment was controlled so that as far as pos-

sible the metabolic state of the animals and the quantity of stored food present were constant, both for individuals within one experiment, and for experiments within a series. Two days before the start of each experiment the required number of *Pelmatohydra* were put into a solution chemically similar to the experimental radioactive solution. This time was more than sufficient for them to reach a steady state as regards internal concentration (see p. 432) so that transfer to the radioactive solution involved no further change in internal concentration, but merely a change in the ratio of isotopes. The volume of external solution was large in relation to that of the experimental animals; therefore, in the belief that the organisms cannot differentiate between isotopes of the same element, it was assumed that in the condition of steady state finally attained, the ratio of the isotopes inside was the same as that outside. This will be true only in so far as the ion under consideration is free to diffuse, and is not chemically bound to some non-ionizing compound. Ignoring any non-diffusable fraction of the element under consideration, the internal concentration may be calculated from the internal radioactivity at steady state, the external radioactivity and the total external concentration of the ion. With this knowledge, and assuming no change in the steady chemical state throughout the experiment, it is also possible to calculate the ionic flux (Davson, 1951), although in fact the variation in the experimental results proved too great for any useful comparisons to be made.

The radioactive isotopes were ^{24}Na, ^{42}K and ^{82}Br, and were obtained in solid form as Na_2CO_3, K_2CO_3, and NH_4Br from the Ministry of Supply, Harwell, and from Oak Ridge, Tennessee. The Na_2CO_3 and K_2CO_3 samples were completely converted to chloride by the addition of HCl and the chloride solutions obtained were then made up to the concentration required for the experiment. In the case of NH_4Br the samples were made up directly as NH_4Br solutions. Some earlier experiments were done with radioactive NaCl and KCl, but these samples were later found to be contaminated with other radioactive isotopes. Therefore, experiments quoted in this paper include only those done with the Na_2CO_3 and K_2CO_3 samples, except in the case of a single experiment shown in fig. 6 when $^{24}NaCl$ was used.

The *Pelmatohydra* were put into the experimental solution after rapid washing in distilled water to remove any traces of salts from the cell surfaces, and at intervals of 15, 30 or 60 min. (whichever was appropriate) two individuals were taken out of the solution and washed quickly in distilled water. The enteron was flushed by inserting a hypodermic needle at the base of the hydra and forcing distilled water through the enteron, and out by the hypostome. Each individual was then placed on a wire hook of known weight, dried for 1½ hr. at approximately 70° C. and weighed with the hook on a quartz fibre balance. Finally, the activity of the whole sample was counted using an end-window counter.

The method of washing was tested by comparing counts of washed and rewashed individuals, and also by washing individuals which had been in a radioactive solution for only 1 or 2 min. During such a short time it was assumed that no appreciable uptake of the tracer would have occurred, and that consequently any counts obtained on such material would be due to insufficient washing. Counts obtained

from these samples were very low and the washing was therefore considered to be adequate.

Errors in counting can occur in a thick sample because radiations from its lower surface are lost by absorption in the sample material. For this reason the method of counting whole hydra rather than homogenized samples (which give an evenly distributed and thin sample symmetrically placed below the counting tube) was checked by counting the whole hydra, and then homogenizing and recounting. No significant difference was found between the counts of whole and homogenized samples, or between counts of samples placed symmetrically and asymmetrically below the counting tube. Theoretical figures obtained from the range-energy relation for β radiation showed that for all three isotopes used the thickness of the hydra samples was below the level where absorption would vitiate the count. It was therefore assumed that there was no need to homogenize the samples.

The results obtained were calculated as count/dry weight/minute (with corrections made for the decay of the radioactivity and for counting rate of the counter). In order to convert these results to counts/mg. of fresh material/minute it was necessary to know the percentage dry weight of the hydra. This was obtained by taking a sample of 20–30 hydra, weighing them wet, and then drying to a constant weight. This was done at regular intervals during the course of the investigation to ensure that any seasonal or other variations were taken into account. In fact these proved to be negligible.

Results

(1) *Viability in experimental solutions*

The effects of various experimental solutions on the viability of *Pelmatohydra oligactis* were investigated by placing the animals in each of a series of these solutions and observing them over a period of 2–3 weeks. From these observations it was found that *Pelmatohydra* will live for approximately 10 days in NaCl concentrations varying from 80 to 8 mg./l., and for 2–3 days in a NaCl concentration of 0·8 mg./l. providing sufficient KCl (i.e. 40 mg./l.) was present. *Pelmatohydra* were also found to live for over 3 weeks in KCl concentrations ranging from 100 to 10 mg./l. and for 10 days in a KCl concentration of 250 mg./l., while the addition of NH_4Br up to a concentration of 50 mg./l. had no effect on the viability of *Pelmatohydra* over a period of 23 days.

It may be mentioned that in all solutions used, both in original experiments and in experiments using radioactive tracers, the pH was approximately 7·2, K_2HPO_4 in the solution acting as a buffer.

(2) *Rate of uptake of ions*

A graph of the uptake of Na is shown in Fig. 3. The equilibrium line is drawn through the mean of all readings obtained after the first 12 hr. of the experiment. Similar graphs were obtained for the uptake of Br and K from solutions containing these isotopes (Figs. 4, 5). From the graphs it is clear that for each isotope a steady state was substantially reached in approximately 12 hr. In the experiments using

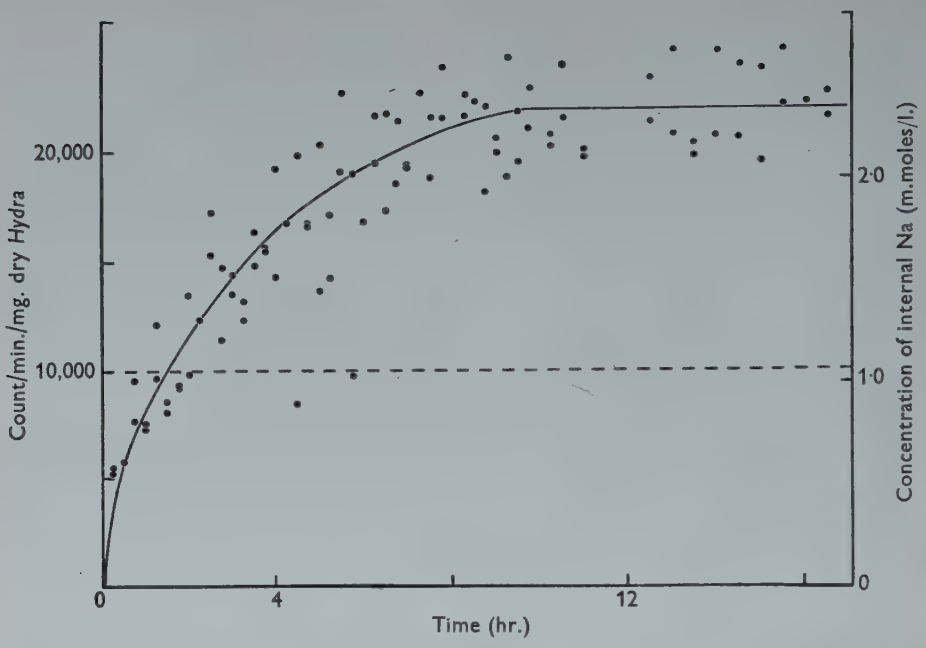

Fig. 3. The uptake of ^{24}Na by *Pelmatohydra oligactis* from a solution containing 1·068 m.moles/l. NaCl. The broken line represents the concentration of ^{24}Na in the external solution expressed as m.moles/l. Na. Other salts present in the solution are 4 mg./l. KCl, 4 mg./l. $CaCl_2$, 4 mg./l. $NaHCO_3$, and 1 mg./l. K_2HPO_4.

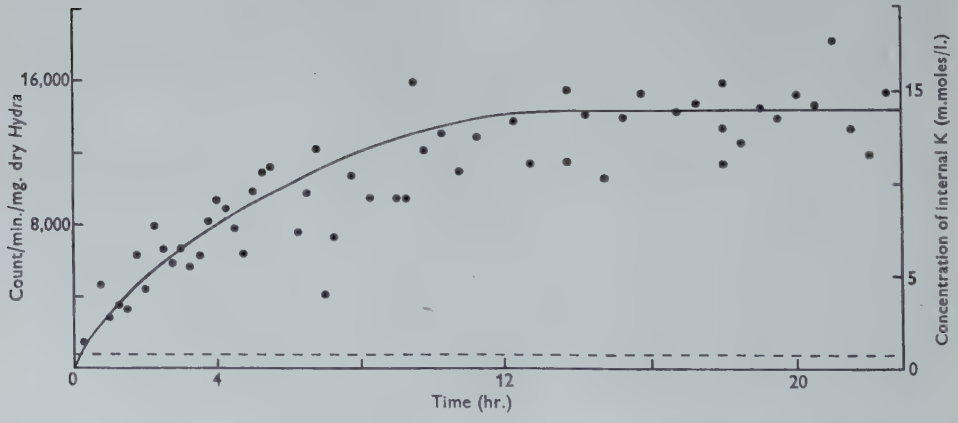

Fig. 4. The uptake of ^{42}K by *Pelmatohydra oligactis* from a solution containing 0·054 m.mole/l. KCl. The broken horizontal line represents the concentration of ^{42}K in the external solution expressed as m.moles/l. Other salts present in the solution are NaCl 40 mg./l., $NaHCO_3$ 4 mg./l., $CaCl_2$ 4 mg./l., and K_2HPO_4 1 mg./l.

^{42}K some values for the internal concentration of K in starved hydra were also obtained. These results (not plotted on the graph) show the same general trend as those obtained for fed hydra, but the scatter of the points is considerably greater, possibly due to the difficulty experienced in washing the enteron of small starved animals. Table 3 gives a summary of results of experiments carried out to show the uptake of Na, Br and K.

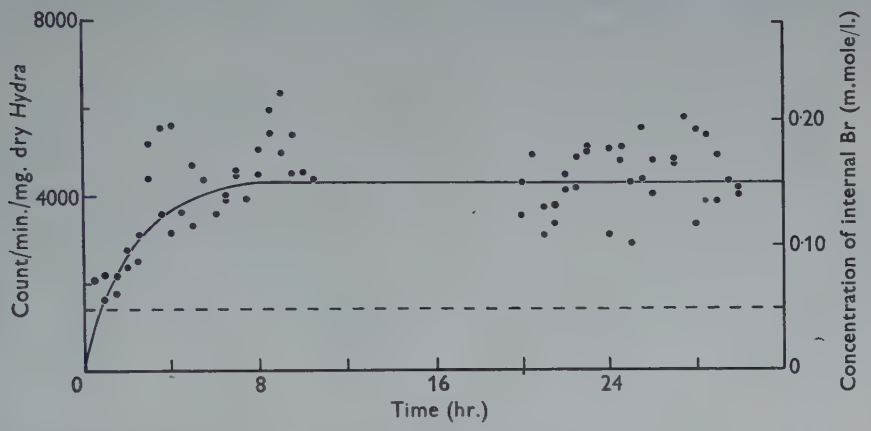

Fig. 5. The uptake of ^{82}Br by *Pelmatohydra oligactis* from a solution containing 0·5 m.mole/l. NH_4Br. The horizontal line represents the concentration of ^{82}Br in the external solution expressed as m.moles/l. Other salts present in the solution are 80 mg./l. NaCl, 4 mg./l. KCl, 4 mg./l. $NaHCO_3$, 4 mg./l. $CaCl_2$, and 1 mg./l. K_2HPO_4.

Table 3

Isotopes	Exp. no.	External concn. (m.moles/l.)	Deduced internal concentration (at equilibrium), in m.moles/l.	Temp. (° C.)
Na	101	1·07	2·34	16·5 –17·75
	104	1·07	3·08	17·75–20·0
Br	1	0·05	0·15	16·0 –16·5
	2	0·05	0·15	16·0 –17·0
	6	0·50	0·15	18·0 –18·5
K	106	0·054	13·8	16·5 –17·0
	107	0·054	15·0	19·0 –19·5

In addition to these experiments, the rate of uptake of ^{24}Na was observed in *Pelmatohydra* which had previously been kept in distilled water until nearly all exchangeable sodium had been washed out. The *Pelmatohydra* were first placed in a solution containing ^{24}Na and left for 16 hr. By this time all exchangeable ^{23}Na was considered to have been replaced by Na from outside the cells (the time taken to reach a steady state in other experiments being approximately 12 hr.), and the animals were transferred to distilled water. The loss of ^{24}Na while the *Pelmatohydra* were in distilled water and the subsequent uptake which occurred when they were transferred back to a solution containing 0·85 m.mole/l. of ^{24}Na is shown in Fig. 6.

In distilled water the internal concentration of ^{24}Na fell almost to zero. Recovery to a level of the same order as that found at the start of the experiment took place on return to a solution containing ^{24}Na. The internal ^{24}Na level in this experiment is higher than in the previous experiments, but the radioactive sample of NaCl used was contaminated with ^{32}P so this higher result is probably due to the presence of ^{32}P. However, although the value for internal Na is not accurate, the experiment does show the ability of *Pelmatohydra* to obtain Na from the external solution when the diffusable Na concentration of the cells is nearly zero.

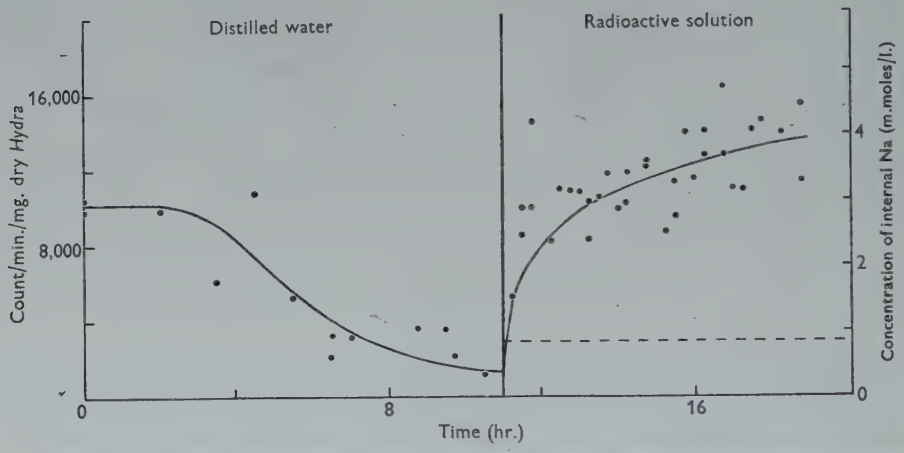

Fig. 6. Loss of ^{24}Na from *Pelmatohydra oligactis* placed in distilled water, and the subsequent uptake of ^{24}Na from a solution containing 0·85 m.mole/l. NaCl. The horizontal broken line represents the concentration of ^{24}Na in the external solution expressed as m.moles/l. Other salts present are $NaHCO_3$ 4 mg./l., KCl 4 mg./l., $CaCl_2$ 4 mg./l., and K_2HPO_4 1 mg./l.

(3) *Relationship of internal and external ionic concentration at a steady state*

Fig. 7 shows the results of an experiment in which *Pelmatohydra* were placed in solutions containing ^{24}Na in concentrations varying from 5·4 to 0·01 m.moles/l. The *Pelmatohydra* were left for 16 hr. before samples were taken in order to ensure that equilibrium had been reached. But after this time samples were taken from each concentration every hour until 10 to 12 samples had been obtained from each solution.

Clearly the internal concentration of sodium was maintained at an approximately constant level of about 2·4 m.moles/l. whilst the external concentration was increased by 250 %. The point marked thus ⊙ on the graph obviously does not agree with the general trend of the results, and has been assumed to be erroneous, and therefore is not included in the calculation of the mean of the points. Results of a second experiment show the same features although the internal level was slightly higher (2·9 m.moles/l.), possibly owing to the fact that the temperature was about 2° C. higher.

Two experiments were carried out with various concentrations of ^{82}Br in the external medium (see Fig. 8). Each point in the graph represents the mean of

samples varying from 6 to 8 in number. In both experiments the internal concentration of bromide was related to the external concentration over the whole range tested. The upper limit of the external concentration of bromide used was set by the results of the viability experiments; *Pelmatohydra* were found to die within 2 days in concentrations of NH_4Br above 2·5 m.moles/l.

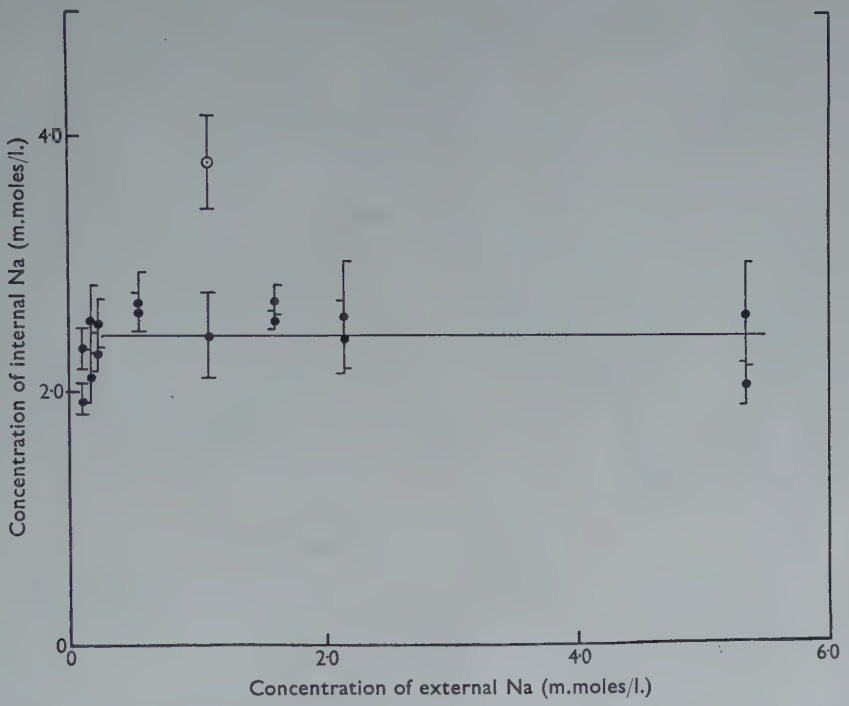

Fig. 7. Graph showing the relationship between the concentration of ^{24}Na in the external solution and in *Pelmatohydra oligactis* after equilibration. The vertical lines through the points indicate the standard error above and below the means. In addition to NaCl the external solution contained the following salts: $NaHCO_3$ 4 mg./l., KCl 4 mg./l., $CaCl_2$ 4 mg./l., and K_2HPO_4 1 mg./l.

Conclusions

The results so far obtained are only in the nature of a preliminary survey, and consequently no definite conclusions can be drawn as to the exact mechanism of ionic control in *Pelmatohydra*. However, from the figures for the total concentration of ions in the external solution, and also of the internal and external concentrations of the radioactive ion under investigation, it is possible to determine whether ions are being concentrated and maintained against a concentration gradient. In the case of Na, K and Br it has been found that the internal concentrations of these ions maintained by *Pelmatohydra* are all well above the concentrations in the external medium (see Figs. 3–5).

The maintenance of an internal concentration of ions above the concentration in the external medium could be explained in various ways. The distribution of K and

Na in muscle fibres was explained in terms of the Donnan equilibrium by Boyle & Conway (1941), although it is now generally agreed that muscle fibres are permeable to sodium.

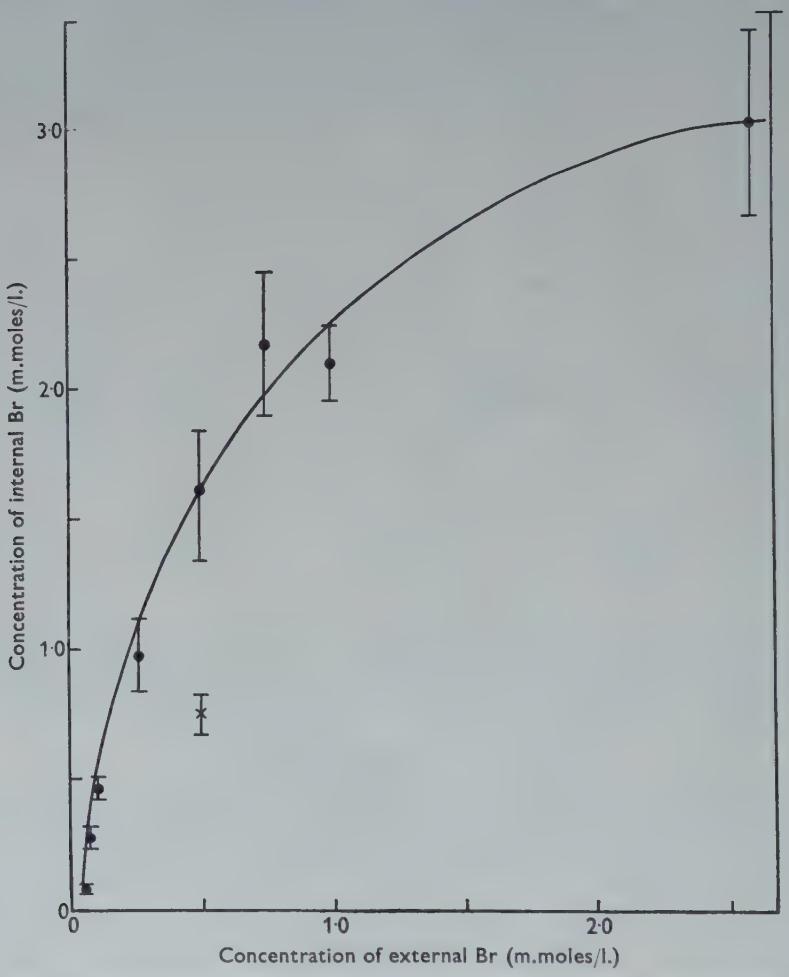

Fig. 8. Graph showing the relationship between the concentration of ^{82}Br in the external solution and in *Pelmatohydra oligactis* after equilibration. The vertical lines through the points indicate the standard error above and below the means. In the case of point X 2·3 m.moles/l. of NH_4Cl were added to the solution. In addition to NH_4Br, the external solution contained the following salts: NaCl 80 mg./l., KCl 4 mg./l., $NaHCO_3$ 4 mg./l., $CaCl_2$ 4 mg./l., and K_2HPO_4 1 mg./l.

The maintenance of high internal concentrations of Na, Br and K in *Pelmatohydra* by a Donnan equilibrium does not seem possible. In order to maintain the high internal K and Na concentrations by a Donnan equilibrium, the Br concentration inside the cell (for experiments in which this was used) should be well below that of the external solution. But the results of the experiment shown in Fig. 8 show the reverse to be true.

Similarly, the steady internal concentration of Na maintained over a wide range of external Na concentrations cannot be interpreted in terms of a Donnan equilibrium; such an equilibrium would demand a steady decrease of internal sodium with a decrease of external sodium, as was found to be the case for potassium in muscle fibres (Boyle & Conway, 1941).

To explain the high internal ionic concentrations maintained by many cells, Ussing (1948) suggested a process of exchange diffusion which is relatively independent of active mechanisms. However, the results of the experiment shown in Fig. 6 indicate that the internal sodium concentration cannot be maintained entirely by exchange diffusion. In this experiment practically all the exchangeable sodium had been washed out of the hydra, but despite this it was regained against a concentration gradient when sodium was added to the external solution.

The results of experiments on anion exchange in *Pelmatohydra* suggest that in this organism chloride and bromide are not distinguished, although further work is needed to establish this fact. The point X in Fig. 8 represents the internal bromide concentration reached when the external solution contained 0·5 m.mole/l. of NH_4Br and 2·2 m.moles/l. of NH_4Cl. The addition of NH_4Cl clearly depressed the internal bromide level reached by the animals, so suggesting that the chloride and bromide are not distinguished by the cells. A similar lack of distinction has been found in other cases, such as mammalian kidney (Frey, 1937).

GENERAL DISCUSSION

The cells of *Hydra viridis* have been shown to be highly permeable to water. The results of experiments determining the volume of ectoderm and endoderm cells in different concentrations of sucrose indicate that the internal osmotic concentration of the animals is above that of the external medium. Under experimental conditions the internal concentrations of sodium, potassium and bromide in *Pelmatohydra oligactis* have been shown to be higher than the concentrations of these ions in the external medium. This will, at least in part, account for the internal osmotic pressure. The osmotic gradient across the cell membrane will tend to drive water from the external solution into the cells, and there must be some mechanism which maintains the hypertonicity of their cellular contents. This mechanism is very probably an active one which could act either by opposing the entry of water into the cells, as has been suggested for *Procerodes ulvae* (Beadle, 1934), or by removing excess water which passes into the cell. Robinson (1950) suggests this latter to be the method by which a constant internal water content is maintained in the rat kidney. At present no definite conclusions can be drawn as to the mechanism controlling the water content of *Hydra*.

An active uptake of ions from the external medium has been demonstrated, although without measurements of potential difference it is not possible to tell whether this uptake is an active uptake of cations or of anions. It is of interest to note that the accumulation of potassium takes place to a higher level than that for sodium, and it is possible that the potassium may be concentrated in the cells while the sodium

is predominantly in the mesogloea. This suggestion has already been put forward by Kitching (1954), where he points out that 'in order to postulate a nerve and muscle physiology comparable with that of higher animals, it would be necessary to suppose this distribution of potassium and sodium, and to attribute to the outermost layer of cells in *Hydra* tissue the power to secrete sodium into the mesogloea or interstitial fluid, after the manner of frog's skin'.

SUMMARY

1. Isolated tentacles of *Hydra viridis* were placed in solutions of sucrose of different known concentrations. In concentrations of sucrose greater than 0·04 M the tentacles shrank, and it was found that the ectoderm, endoderm, and enteron all contributed to this shrinkage. Both ectoderm and endoderm are highly permeable to water. Calculations suggest that the ectoderm cells are at least as permeable as various other cells which have been investigated by many workers.

2. Shrinkage of the tentacle was very slight in 0·05 M sucrose, and no shrinkage occurred in 0·04 M sucrose. It is therefore suggested that the internal osmotic pressure is equivalent to that of 0·04–0·05 M sucrose.

3. The uptake of the radioactive ions ^{24}Na, ^{82}Br, and ^{42}K by *Pelmatohydra oligactis* has been investigated. All these ions are concentrated above the levels in the external medium.

4. The relations between internal and external concentrations have been determined for sodium and bromide. The internal sodium concentration is maintained at a steady level with external concentrations varying from 5·4 m.moles/l. to approximately 0·20 m.mole/l. The internal bromide level has been shown to fall as the external bromide decreases from 2·5 to 0·05 m.mole/l. Higher external bromide concentrations could not be used as the animals were not healthy in solutions containing more than 0·25 g./l. NH_4Br. The internal bromide is also shown to be depressed by the addition of chloride to the external solution.

5. The results obtained indicate that the cells of *Hydra* are highly permeable to water, and also capable of maintaining an internal concentration of certain ions well above that of the external medium. This requires some form of regulation of water content and of ionic content. In the case of the latter it is not at present possible to say whether it is an active uptake of cations or anions.

I am greatly indebted to Dr J. A. Kitching, both for suggesting this problem to me, and for his helpful advice and criticism; also to Prof. J. E. Harris and Prof. M. Gardiner in whose departments this work was carried out.

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A STUDY OF THE BLOOD GROUPS OF THE RABBIT, WITH REFERENCE TO THE INHERITANCE OF THREE ANTIGENS, AND THE AGGLUTINABILITY OF THE RED CELLS CARRYING THEM

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INTRODUCTION

Work on rabbit blood groups carried out by several workers prior to 1950, has been summarized by Heard (1953). The sera, however, are not available and the rabbits possessing the antigens have died, so that the direct correlation between previous and present work has been impossible.

Kellner & Hedal (1953), working in America, have described an allelomorphic pair of blood group genes, *G* and *g*; while Heard (1953) in this laboratory has identified and described four blood group antigens, namely *Z*, *Y*, *X* and *W*. Heard found that red blood cells of rabbits possessing a particular antigen may be agglutinated to varying titres by an antiserum containing a single antibody corresponding to that antigen. Cells which were able to demonstrate antibody to a high titre she called 'strong', while those that demonstrated the same antibody only to a low titre she called 'poor'. She brought forward evidence to demonstrate that 'strong' cells possess more functional antigen sites than 'poor' cells.

In the present work Heard's anti-*Z* and anti-*Y* have been compared with Kellner & Hedal's anti-*G* and anti-*g*. Antigens *Z* and *G* were found to be identical, as were *Y* and *g*.

A study of the inheritance of the antigens *Z*, *Y* and *W*, has been undertaken, and evidence is brought forward proving that these antigens are controlled by an allelomorphic trio. The degree of agglutinability of the cells by two of the antibodies concerned has been shown to be related to the genotype of the rabbit bearing the cells.

Finally a notation is suggested which will correlate the antigenic systems of Heard and Kellner & Hedal.

TECHNIQUE

Reagents

(1) *Anticoagulant.* A solution of 0·6% tri-sodium citrate in 3·3% aqueous magnesium sulphate solution was used as an anticoagulant for bleedings of small volume. For larger volumes (20–40 ml.) 3·4% tri-sodium citrate solution was used.

(2) *Medium in which tests were performed.* Lawrence (unpublished) showed that a non-specific agglutination sometimes occurs when saline is used as a suspending

medium for rabbit red cells. Heard, Hinde & Mynors (1949) found that 3·3% aqueous solution of magnesium sulphate was a satisfactory medium which prevented such anomalies, and this has been used throughout the present work.

(3) *Red cell suspension.* The standard suspension of erythrocytes used was 2% by volume of packed cells. This was made up by matching the density of suspension with an accurately measured 2% suspension of sheep cells.

(4) *Anti-rabbit globulin serum.* The goat anti-rabbit globulin serum used in this work was obtained from a goat which had received five courses of injections of rabbit serum. The antiserum after heat inactivation was absorbed with well-washed rabbit red cells in order to remove agglutinins to unsensitized rabbit red cells. The antiserum had a titre of 64 when tested against suitably sensitized rabbit cells. The serum was normally used at a dilution of 1/25.

(5) *Test tubes.* Tests were performed in glass tubes of two types: (*a*) 8 × 55 mm. called C.F. tubes in this laboratory; (*b*) 5 × 50 mm. called R.H. tubes in this laboratory.

Direct agglutination tests

Dilutions were performed with a graduated 1 ml. syringe in C.F. tubes. Serum dilutions were distributed in aliquots of 0·1 ml., to which 0·1 ml. of red cell suspension was added. The tubes were shaken, and two drops of the serum-cell suspension transferred to an R.H. tube, and incubated at 37° C. for 1 hr.

Readings were made macroscopically, and microscopically by spreading the cells on a slide and examining under the low power of a microscope. In the event of there being discrepancy between the two readings, the microscopic reading was taken as being the more reliable.

Anti-globulin sensitization test

The anti-globulin sensitization test was always performed in parallel with a direct agglutination titration. The serum cell suspension left in the C.F. tubes (after removal of the two drops for the direct agglutination test) were incubated for 1 hr. at 37° C., washed 3 times in magnesium sulphate solution, and resuspended to give a concentration of approximately 2%. One drop of the sensitized cells was added to one drop of goat anti-rabbit globulin serum in an R.H. tube, and incubated for 30 min. at 37° C. The test was read macroscopically and microscopically, but in this case the macroscopic reading was taken as being the more reliable, since the agglutinates are very fragile.

Blood grouping tests

'Grouping' tests were always performed in R.H. tubes using serum at a dilution well below its titre. One drop of cell suspension was mixed with one drop of serum, and incubated for 1 hr. at 37° C.

The antiglobulin sensitization test was performed and readings were taken as previously described.

Absorption

Blood to be used for absorptions was bled into 3·4 % tri-sodium citrate, centrifuged, the supernatant fluid removed, and the cells washed twice in the suspending medium. At the last wash the cells were centrifuged for 15 min. and the supernatant fluid removed.

The required volume of packed cells was then transferred to a tube containing the suspending medium. The cells were resuspended, and repacked for 15 min. in the centrifuge. The supernatant fluid was removed immediately prior to the addition of serum to be absorbed. All standard serum absorptions were made with equal volumes of packed cells, and were performed 6 times, or until absorption was complete.

ANALYSIS OF RABBIT ISO-ANTISERA OBTAINED FROM DRS KELLNER AND HEDAL

Dr A. Kellner and Dr E. F. Hedal kindly sent to this laboratory samples of their rabbit iso-antisera anti-*G* and anti-*g*. These antisera were known to have a direct agglutination titre of 256 (anti-*G*) and 500 (anti-*g*). The anti-*G* serum was known to contain another unnamed antibody, in the incomplete form, of different specificity from either anti-*G* or anti-*g*. In order to avoid complications due to this incomplete antibody only direct agglutination reactions were used at this stage of the work.

The cells of forty-four members of a panel of rabbits of mixed breed were tested by direct agglutination against these sera at a dilution of 1 10 (Table 1).

Table 1. *The numbers of animals which give positive reactions with antisera to Z and G, and Y and g*

	Z+	*Z*−		*Y*+	*Y*−
G+	27	3	*g*+	21	0
G−	0	14	*g*−	0	23

It may be seen that the distribution of animals positive to antisera *G* and *g* is almost identical with those positive to Heard's (1953) two antisera *Z* and *Y* respectively. There were, however, three animals (B 59; A 17 and 1535) whose cells were very weakly positive to serum anti-G and yet negative to serum anti-*Z*. Nevertheless, the evidence detailed below points strongly to *Z* being identical with *G*, and similarly *Y* with *g*.

Kellner & Hedal's conclusion (1953) that all rabbits possess either *G* or *g* was found not to be true of the animals tested in this laboratory.

Of the seven animals in the panel which were negative to antisera *Z* and *Y*, four were also negative to both anti-*G* and anti-*g*, while the other three were responsible for the exceptionally weak reactions with anti-*G*, discussed above. In order to confirm that *G* and *g* may both be absent from a rabbit, *Z*-negative, *Y*-negative animals

were mated. It was confirmed that the litter was $Z-Y-$ and then the eight young were tested against anti-*G* and anti-*g*.

It may be seen that three members of the litter gave unexpected positive direct agglutination reactions with serum anti-*G* (Table 2).

Table 2. *The reactions of a Z-negative, Y-negative (i.e. ww) litter with unabsorbed antisera to G and g*

Member of $Z-$ $Y-$ litter	Anti-*G*	Anti-*g*
♂1	−	−
♂2	++	−
♂3	−	−
♂4	+	−
♂5	−	−
♂6	−	−
♂7	+	−
♂8	−	−

It seemed possible that the difference of reaction between anti-*G* and anti-*Z* might be due to the unnamed incomplete antibody known to be in the anti-*G* serum, perhaps acting as a complete antibody to some cells.

To test this hypothesis the following cells were used to absorb different portions of serum anti-*G*:

(*a*) *A*45 which reacted to the unnamed incomplete antibody in anti-*G* but did not possess the *G* antigen itself.

(*b*) ♂2 of the litter previously described. This was negative to sera both anti-*Z* and anti-*Y*, but gave positive direct agglutination with serum anti-*G*.

(*c*) *A*16 which possessed no antigen reacting with anti-*G* and was used as a control.

Testing back produced the results shown in Table 3.

It may be seen that both ♂2 and *A*45 cells removed the 'impurities' reacting with *A*45, ♂2, ♂4 and ♂7, but left the characteristic anti-*G* agglutinin, which then had reactions identical with anti-*Z*. The three exceptional cells (*A*17, 1535 and *B*59) which gave rise to the discrepancy between *Z* and *G* in Table 1 all gave negative reactions with direct agglutination and antiglobulin sensitization test with the absorbed anti-*G* sera. The very weak reaction with ♂2 cells shown by anti-*G* absorbed by *A*45 could not be removed by further absorption with *A*45. This weak reaction could not be demonstrated by the antiglobulin sensitization test, as presumably the antibody was washed off the cells. In this it is quite different from the main antibody reacting with ♂2 and *A*45, and must represent a very weak antibody in the anti-*G* serum of a third specificity.

It seems likely that ♂2 and *A*45 bear the same antigen, but this could not be fully established since further absorptions could not be done owing to lack of serum. Serum anti-*G*, therefore, contains the typical *G* agglutinin and at least one other agglutinin which probably acts as an incomplete antibody with some cells and a

Table 3. *The reactions of serum anti-G, after absorption with Z-negative cells which had shown a reaction with the unabsorbed serum*

Serum anti-*G* ↓	Method of testing	*Z*-negative cells								Five other *Z*− cells	Twelve *Z*+ cells
		*A*45	*J*2	*J*4	*J*7	*B*59	1535	*A*17	*A*16		
Unabsorbed	*DA*	—	++	+	+	*w*	(+)	(+)	−	−	++
	AG	(++)	++	++	++	++	++	++	−	2−, 3+	++
Absorbed by *A*16 control	*DA*	−	(++)	+	(++)	−	−	(*w*)	.	−*	++†
	AG	++	++	++	++	++	++	++	.	++*	++†
Absorbed by *A*45	*DA*	−	*w*	−	−	−	−	−	.	−	++
	AG	−	−	−	−	−	−	−	.	−	++
Absorbed by *J*2	*DA*	−	−	−	−	−	−	−	.	−	++
	AG	−	−	−	−	−	−	−	.	−	++

DA = direct agglutination; *AG* = antiglobulin;
* = Only two animals tested; † = Only four animals tested.

complete antibody with others. It also contains a trace of antibody of a third specificity.

In further experiments, whose object was to verify that *G* and *Z*, and *g* and *Y*, were identical, titrations were performed in parallel, to see whether the cells which are less agglutinable for *Z* were also less agglutinable for *G*, and similar comparisons were made between *Y* and *g*.

Table 4. *The parallelism in titres obtained with antisera to Z and G; and Y and g*

Test cells	Anti-*Z* titre	Anti-*G* titre	Test cells	Anti-*Y* titre	Anti-*g* titre
2403	16	32	1963	32	16
2472	32	64	2472	32	64
A21	32	64	A25	64	64
A25	64	128	B66	256	256

The close parallelism between these results provides additional evidence that *Z* is identical with *G*, and *Y* with *g*.

Kellner & Hedal's experiments on haemolysis of rabbit cells by anti-*G* and anti-*g* in the presence of guinea-pig complement have been repeated and confirmed.

Kellner & Hedal (1953) described *G* and *g* as an allelic pair, at least one of which must be present in every rabbit. While their evidence seems to show this to have been the case with their own rabbits, it is not true of the stock of this laboratory in which a number of rabbits have been found which possess neither *G* (*Z*) nor *g* (*Y*). These animals have been found to possess Heard's antigen *W*.

GENETIC RELATIONSHIP BETWEEN ANTIGENS *Z*, *Y* AND *W*

If it is assumed that *Z*, *Y* and *W* are inherited independently, the eight genetic combinations possible are shown in Table 5.

Table 5. *The distribution of rabbits possessing the antigens Z, Y and W*

Z	*ZY*	*ZW*	*Y*	*YW*	*W*	*ZYW*	o
22	19	19	4	11	18	o	o

It may be seen that among ninety-three members of the panel, all had at least one, but never more than two of the three antigens. This distribution suggested that *Z*, *Y* and *W* formed an allelomorphic trio, and therefore breeding experiments were performed in order to test this hypothesis. Twenty-one types of mating are possible on this hypothesis, and all these have been tested.

Among the sixty-two families investigated, with 258 young, not a single example has been found where antigens present in the young were incompatible with those expected from their known parentage (Table 6).

The antigens *Z*, *Y* and *W* behave as Mendelian characters controlled by three allelomorphic genes; the characters cannot strictly be called dominant, for the heterozygote has somewhat less antigen than the homozygote.

Table 6. *The inheritance of the antigens Z, Y and W. Table of matings*

Type of mating	Number of families tested	No. of babies in litters of genotypes					
		ZZ	*ZY*	*ZW*	*YY*	*YW*	*WW*
ZZ × *ZZ*	8	29	.	.	.	.	.
ZZ × *ZY*	4	6	10	.	.	.	.
ZZ × *ZW*	5	6	.	6	.	.	.
ZZ × *YY*	2	.	12	.	.	.	.
ZZ × *YW*	1	.	1	5	.	.	.
ZZ × *WW*	1	.	.	6	.	.	.
ZY × *ZY*	3	1	8	.	5	.	.
ZY × *ZW*	3	1	4	2	.	3	.
ZY × *YY*	3	.	7	.	8	.	.
ZY × *YW*	1	.	1	0	4	0	.
ZY × *WW*	5	.	.	11	.	11	.
ZW × *ZW*	3	5	.	2	.	.	1
ZW × *YY*	3	.	6	.	.	6	.
ZW × *YW*	4	.	2	0	.	4	6
ZW × *WW*	5	.	.	11	.	.	15
YY × *YY*	1	.	.	.	4	.	.
YY × *YW*	3	.	.	.	6	8	.
YY × *WW*	1	.	.	.	.	3	.
YW × *YW*	1	.	.	.	1	4	1
YW × *WW*	2	.	.	.	.	4	4
WW × *WW*	3	.	.	.	.	.	18

DOSAGE EFFECT OF GENES OF THE *Z*, *Y* AND *W* SYSTEM

As a result of direct agglutination titrations on rabbit cells Heard (1953) showed that:

(*a*) Cells bearing the same antigen were agglutinated to different titres by a serum containing a single antibody. Those cells that demonstrated antibody to a high titre were said to be 'strongly agglutinable', and those that were able to demonstrate the same antibody to a low titre were said to be 'poorly agglutinable'.

(*b*) 'Strongly agglutinable' cells appear to have a larger number of functional antigenic sites on the erythrocytes than 'poorly agglutinable' cells as demonstrated by absorption experiments.

Examination of the data for *Z* and *Y* suggested that the least agglutinable cells are those with the genotypes *ZW* and *YW*, and the most agglutinable cells are homozygous for *Z* and *Y*. In order to investigate this possible relationship between the genotype of a cell, and its degree of agglutinability, a series of direct agglutination titrations was performed for *Z* and *Y* antigens. The antiglobulin test was not used in these titrations, as it was found that the titre of a serum differed little between different types of cells when the antiglobulin sensitization test was performed. Titrations have not been performed for *W*, as no high titred 'complete' anti-*W* sera are yet available.

The titrations were performed by the method outlined in the section on technique. At each titration at least one constant control cell was used, namely a very poorly agglutinable cell, 54 for *Y* and 2473 for *Z*.

Using a modified form of Race & Sanger's (1950) method of scoring, the titration readings were converted into numerical values, i.e.:

$$++V=10, \quad ++=8, \quad (++)=6,$$
$$+=5, \quad (+)=3, \quad W=2, \ (W)=1.$$

The values obtained in each titration were totalled to give a 'score' for each type of cell. To prevent the scores from becoming too large the values below a fixed base-line were ignored. This base-line was taken as the (++) end-point for the standard poorly agglutinable type of cell. An example of scoring is shown in Table 7.

Table 7. *The method of scoring used in titrations*

Cells	Serum dilutions								Score
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
54	++	++	(+)	(*w*)	–	–	–	–	12
12	++*v*	++*v*	++*v*	++*v*	++*v*	(++)	*w*	–	48
				↑ base-line					

Each titration was performed at least twice, and the values obtained were averaged for each type of cell. In the case of the control cells the maximum discrepancy obtained between repeated titrations was 5 points for *Z* and 3 points for *Y*, whereas the greatest discrepancy for the cells of any rabbit was 12 for *Z* and 11 for *Y*. Therefore the greatest error in this work represents a difference in titre of slightly more than one doubling dilution. The average values were plotted on a graph (Fig. 1).

It may be seen that in the case of the *Y* antigen the range of scores shown by the *YY* cells, which are the most agglutinable, does not overlap that of the *YW* cells, which are the least agglutinable. The *YZ* cells occupy an intermediate position between the *YY* and *YW* cells, and overlap the range of *YW* cells.

The relationship is not so clear with regard to the *Z* antigen. There appears to be little, if any, difference between the ranges of *ZY* and *ZW* cells, although *ZZ* cells are clearly more agglutinable than either.

If we accept Heard's (1953) conclusion that strongly agglutinable cells in the rabbit have a large number of antigen sites, then it appears that the cells homozygous for *Z* and *Y* possess more *Z* and *Y* antigen sites than the corresponding heterozygous cells. Furthermore, in cells heterozygous for *Y*, most *YZ* cells have more *Y* sites than *YW* cells.

Similar 'dosage' effects have been recognized in human blood groups in the *MNSs*, '*Rh*', Kell, Duffy and *P* systems (Landsteiner & Levine, 1927; Sanger &

Race, 1951; Race, Taylor, Boorman & Dodd, 1943; Lawler & Race, 1950; Malone & Dunsford, 1951; Race, Sanger & Lehane, 1952; Plant, Ikin, Mourant, Sanger & Race, 1953; Fisher, 1953; Mourant, 1947; Race, 1953).

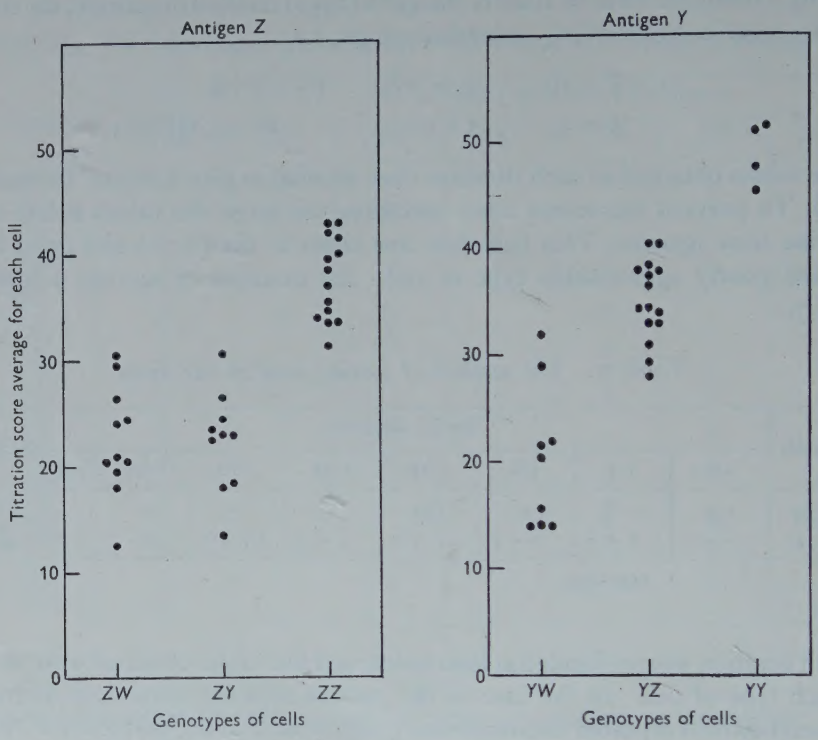

Fig. 1. Graph to illustrate the differences in titre obtained by titration of anti-*Z* and anti-*Y* sera with cells of different types

The effect here seems analogous to that in the *Rh* system in man, where *cDE/cde* cells are more agglutinable with anti-*E* sera than *CDe/cDE* cells, where possibly the homozygous *D* competes for substrate with the *E* antigen more successfully than does heterozygous *D* (Lawler & Race, 1950). Similarly, in rabbits *W* appears to compete with *Y* for substrate more effectively than does *Z*, and therefore the number of *Y* sites is lower in *YW* than *YZ* cells.

NOTATION

Heard's temporary notation of *Z*, *Y* and *W* is not the most convenient way to express an allelomorphic trio. The notation of Kellner & Hedal (1953) has precedence over that of Heard, but the use of *G* and *g* leaves difficulty as to the naming of the third allele. It is therefore suggested that the alleles be called: G^a (identical with *G* and *Z*); G^b (identical with *g* and *Y*); G^c (identical with *W*).

The use of this system leaves room for the addition of any further alleles that may subsequently be found.

It seems possible that G^a, G^b, and G^c may be the same as Castle & Keeler's (1933) allelomorphic trio, $H1$, $H2$, and O.

G^c can most probably be identified with O since complete antibodies to G^c are rare, and therefore this antigen would not have been easily demonstrable before the advent of the antiglobulin sensitization test. It has not been possible to compare gene frequencies with those of other authors, due to deliberate selection and inbreeding in the present stock.

SUMMARY

1. Kellner and Hedal's antigen G is identical with Heard's antigen Z, and similarly g with Y.

2. Kellner & Hedal's (1953) conclusion that all rabbits must possess at least one of the antigens G or g was found not to be true of animals in this stock. Animals lacking both antigens were found to possess Heard's antigen W.

3. Z, Y and W form an allelomorphic trio.

4. Heard's evidence strongly suggests that the most agglutinable cells have more antigen sites (for the antibody in question), than less agglutinable cells. Evidence provided here shows that in the case of the Z and Y antigens homozygous cells are more agglutinable than heterozygous cells, and therefore probably have more sites of the Z or Y antigens respectively. Also the number of Y antigen sites is greater in YZ than in YW cells. It was not possible to demonstrate a similar difference for Z between ZY and ZW cells.

5. It is suggested that the notation of this allelomorphic trio in rabbits should be standardized as follows: G^a = Kellner & Hedal's G and Heard's Z; G^b = Kellner & Hedal's g and Heard's Y; G^c = Heard's W.

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ADDENDUM

Since the completion of this paper, the fifty-three offspring of thirteen more matings in ten of the mating categories shown in Table 6 have been tested. All offspring of these matings possessed the expected Z, Y and W antigens.

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